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Spectrophotometric determination of calcium, magnesium and inorganic phosphate in cerebrospinal fluid.

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SPECTROPHOTOMETRIC DETERMINATION OF CALCIUM, MAGNESIUM AND INORGANIC PHOSPHATE IN CEREBROSPINAL FLUID

by

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A Thesis
submitted to the Faculty of Graduate Studies through
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ABSTRACT

SPECTROPHOTOMETRIC DETERMINATION OF CALCIUM, MAGNESIUM AND INORGANIC PHOSPHATE IN CEREBROSPINAL FLUID

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The aim of this study was to find a simple and rapid spectrophotometric method for the microdetermination of calcium, magnesium and inorganic phosphate in cerebrospinal fluid. Cresolphthalein complexone, Calmagite and Methylthymol blue were studied under similar conditions for calcium, while Magon, Calmagite and Methylthymol blue were examined under a different set of conditions for magnesium. Inorganic phosphate determination was done by the phosphomolybdate technique.

The preliminary investigations dealt with interference studies for calcium and magnesium. EGTA and 8-hydroxyquinoline were used to chelate calcium and magnesium, respectively. Pooled cerebrospinal fluid was used throughout the clinical studies. Precision, sensitivity, specificity, and recovery studies were investigated for each reagent.

Cresolphthalein complexone was found to be the most sensitive and specific reagent for calcium determination

while Magon was highly suitable for magnesium determination in cerebrospinal fluid. Similarly, the phosphomolybdate method was highly sensitive for phosphate determination in cerebrospinal fluid.

DEDICATION

To my Mom and Dad, Donald, Wendy and Geraldine,
with warmest love and profound gratitude.

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In addition, I would like to thank Mrs. Zeleney for her patience and excellent typing of this manuscript; to Frances Komon, for her friendship, encouragement and moral support during the completion of this thesis; and finally, to my parents and family for their understanding and the opportunity to pursue my dreams.

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LIST OF ABBREVIATIONS

A	Absorbance
ACD	Arsenite-citrate reagent
AMA	Ammonium molybdate-acetic acid
A-SDS	Ascorbic acid-sodium dodecyl sulfate reagent
CSF	Cerebrospinal fluid
CPC	o-Cresolphthalein complexone
C.V.	Coefficient of variation
DEA	Diethylamine
dL	Deciliter
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis-(2-aminoethyl)NN N',N'-tetraacetic acid
g	Gram
L	Liter
µL	Microliter
mEq	Milliequivalent
mg	Milligram
mL	Milliliter
mM	Millimole per liter (millimolar)
mmol	Millimole
MTB	Methylthymol blue
M.W.	Molecular weight
n	Number of samples

nm	Nanometers
ppm	Parts per million
r	Correlation coefficient
S.A.S.	Statistical Analysis System
S.D.	Standard deviation
SDS	Sodium dodecyl sulfate
S.E.M.	Standard error of the mean
λ	Wavelength

CHAPTER I

INTRODUCTION

A. CEREBROSPINAL FLUID

1. Origin

The human cerebrospinal fluid (CSF) is a clear, colourless liquid found in the ventricles of the brain and in the subarachnoid space that surrounds both brain and spinal cord (1,2). The CSF also occupies the cisterns which are essentially dilatations of the subarachnoid space (between the arachnoid membrane and pia mater).

The choroid plexuses situated in the ventricles of the brain are generally considered to be the main sites for the production of CSF (1,3). Choroid plexuses are located in the temporal regions of the lateral ventricles, the posterior region of the 3rd ventricle and also in the roof of the 4th ventricle. These highly vascular structures are made up of tiny pouches which are composed of pia mater, capillaries and the ependymal cells lining the ventricles and projecting into them. Each choroid plexus contains an afferent arterial and efferent venous circulatory system (3).

Cerebrospinal fluid is being continuously secreted into the ventricles and reabsorbed at approximately 750 mL per day (1,4,5). The net pressure is regulated by both these factors and the average in a normal supine person is about 160 mm H_2O (12 mm Hg).

Figure 1 illustrates the origin and circulation of the CSF. Originating from the lateral ventricles, the fluid goes through the foramina of Monroe into the 3rd ventricle which produces more fluid. The CSF then moves through the aqueduct of Sylvius into the 4th ventricle where more fluid is produced. The combined fluids then go through the 2 lateral foramina of Luschka and the medial foramen of Magendie into the cisterna magna. Thence, the CSF flows upward to the arachnoid villi of the cerebrum through the subarachnoid spaces, where it is reabsorbed (3).

The net flow is from the cerebral ventricles to the surface of the cerebral hemispheres (6,7). Bering in 1955 (8) found that fluid secreted within the ventricles is actively pumped out of the ventricular system by the choroid plexuses. Thus the fluid is formed and absorbed throughout the ventricles and subarachnoid spaces, and is kept in constant motion by the pulsation of the choroid plexus, seemingly progressing to the surface of the cerebral

FIGURE 1

ORIGIN OF CEREBROSPINAL FLUID

Legend

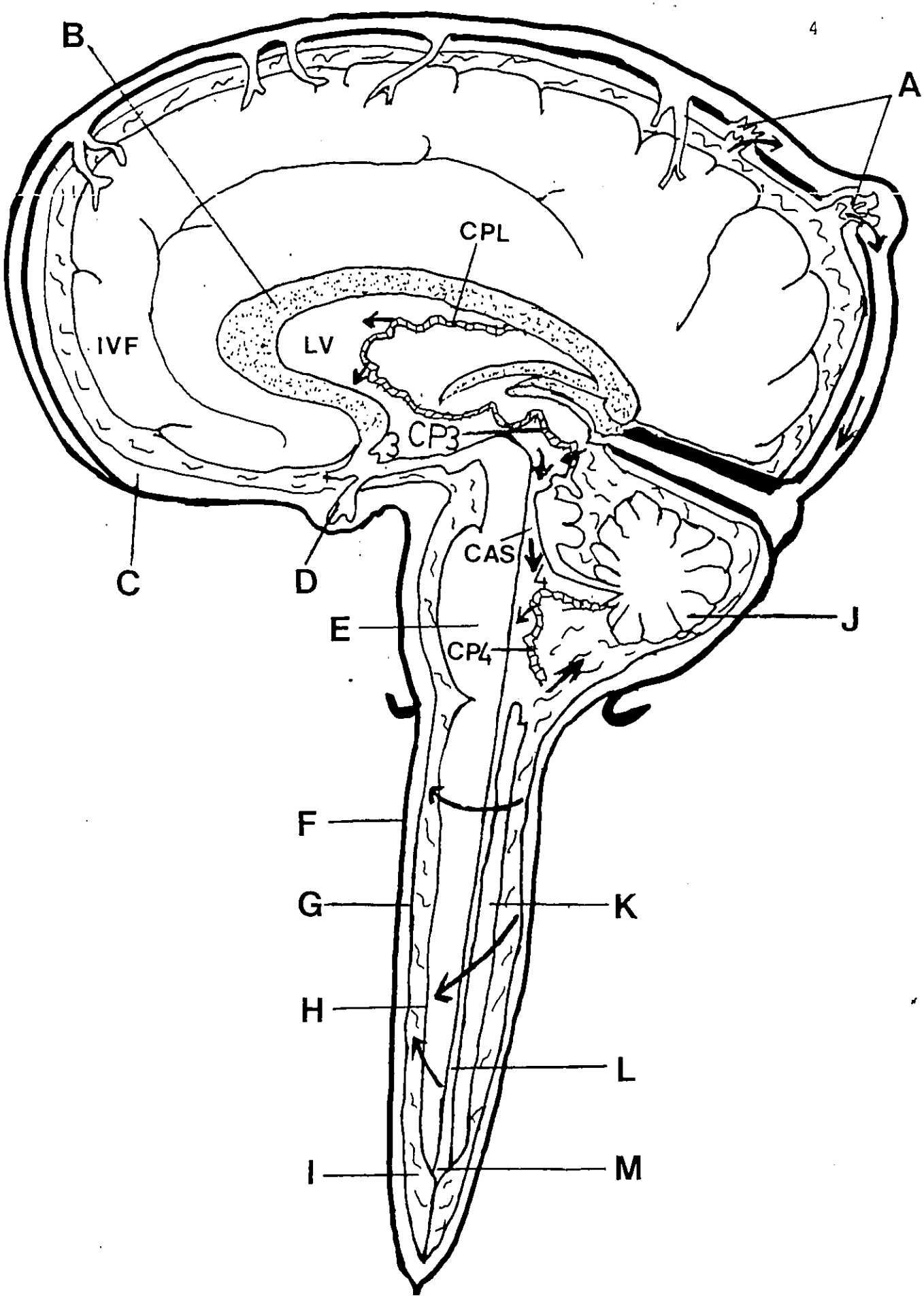
The human brain and spinal cord is depicted in the figure. Arrows show the flow of CSF.

A: Arachnoid villi
B: Corpus callosum
C: Subdural space
D: Hypophysis
E: Pons
F: Dura mater
G: Arachnoid
H: Pia mater
I: Subarachnoid space
J: Cerebellum
K: Spinal cord
L: Central canal
M: Terminal ventricle

IVF: Interventricular foramen of Monroe
LV: Lateral ventricle
3: 3rd ventricle
4: 4th ventricle
CPL: Choroid plexus of lateral ventricle
CP3: Choroid plexus of 3rd ventricle
CP4: Choroid plexus of 4th ventricle
CAS: Cerebral aqueduct of Sylvius

(Adapted from Jensen, D. (1980) The Human Nervous System, p. 10, Appleton Century-Crofts, New York).

FIGURE 1



hemispheres (9)

2. Physiology

The cerebrospinal fluid acts as a cushion for the brain as both brain and fluid have the same specific gravity of 1.004 to 1.008 (2). The CSF pressure depends on two factors, namely, the relative rates of secretion and absorption of CSF. The process of osmosis due to the low protein content is reponsible for absorption (1,2). Certain factors can change the pressure, e.g., elevation of pressure is caused by an increase in CSF volume or obstruction in sinuses of the brain whereby free absorption is blocked (9). Anaesthetics and carbon dioxide can cause a rise in pressure through either primary or secondary changes. Heisey et al. (10) showed that CSF production is independent of hydrostatic pressure, which has been confirmed by others (11).

Thus, it would seem that about 60% of the CSF is formed in the ventricles and 40% in the subarachnoid space (9).

3. Composition and Biochemistry

The composition of cerebrospinal fluid was described by de Rougemont et al. (4). This differs somewhat from plasma; sodium content is about 7% higher, glucose is 30% lower while potassium is 40% lower than that in plasma.

In normal cases, there would be no bilirubin in CSF (12) and the amino acids concentration would only be 30% of blood level. Thus, CSF is not just a plasma filtrate but a secretion (3).

High concentrations of sodium and magnesium ions in CSF suggest active transport from blood to CSF while lower concentration of potassium indicates the reverse process (9). The choroid epithelial cells are responsible for the secretion of sodium into CSF by an active transport mechanism. A net positive charge is maintained which causes chloride and other anions to move passively into the CSF. A comparison of the average concentrations of solutes between CSF and plasma is given in Table I.

It can be seen from Table I that magnesium in CSF (13) is slightly higher than in plasma whereas the reverse is true for calcium (13) and inorganic phosphorus. The normal range of calcium concentration in CSF is quoted as 2.1 - 2.7 mEq L^{-1} (13-15) or 0.975 - 1.3 mmol L^{-1} Ca (3.9 - 5.1 mg/dL) while that of magnesium is 2.0 - 2.7 mEq L^{-1} (mmol L^{-1} $Mg_{1/2}^{2+}$) or 1.0 - 1.35 mmol L^{-1} Mg (16,17).

In 1955, Harris and Sonnenblick (18) found the mean level for calcium to be 4.95 mg % ± 0.11 and the mean level for Mg to be 3.0 mg % ± 0.006. Merritt and Fremont-Smith

TABLE I

AVERAGE CONCENTRATION OF SOLUTES IN CSF AND PLASMA[a]

Solutes	CSF	Plasma
Na^+ (mEq L^{-1})	147.0	150.0
K^+ (mEq L^{-1})	2.9	4.6
Ca^{2+} (mEq L^{-1})	2.3	4.7
Mg^{2+} (mEq L^{-1})	2.2	1.6
Cl^- (mEq L^{-1})	113.0	99.0
HCO_3^- (mEq L^{-1})	25.1	24.8
Glucose (mg %)	64.0	100.0
Inorganic P (mg %)	3.4	4.7
Lactic acid (mg %)	18.0	21.0
Protein (mg %)	20.0	6000.0
pH	7.3	7.4
pCO_2 (mm Hg)	50.2	39.5
Osmolality (mOsm/kg H_2O)	298.0	289.0
Urea (mg %)	12.0	15.0

[a]Adapted from Jensen, D. (1980) The Human Nervous System, p. 112, Appleton Century-Crofts, New York.

(14) in 1937 have reported a range of 4.5 - 5.5 mg % for Ca while Flexner (19) has reported a range of 4.0 - 7.0 mg % for Ca and 3.04 - 3.65 mg % for Mg. On the other hand, Cohen (20) has given a range of 1.0 - 3.5 mg % for Mg. The results of Stutzman and Amatusio (15) namely, 4.86 ± 0.13 mg % for Ca and 2.88 ± 0.17 mg % for Mg agrees well with the investigations of Harris and Sonnenblick (4.95 ± 0.11 mg % for Ca and 3.01 ± 0.06 mg % for Mg) (18). The ratio of Ca^{2+}/Mg^{2+} is distinctly constant with a value of 1.64 ± 0.03 (18,21) which coincides with a value of 1.60 ± 0.06 found by Stutzman and Amatusio (15). The ratio is constant regardless of the absolute amounts of calcium and magnesium.

Bradbury et al. (22) in 1968 found the concentration of Ca in CSF to be 5.2-6.0 mg % by atomic absorption.* Tietz (23) gave a range of 2.1 - 2.7 mmol $Ca_{1/2}^{2+}\ L^{-1}$. In general, the concentration of Ca in the CSF is less than in plasma (1). This is more pronounced if one takes into account ionized Ca^{2+} instead of total Ca. The concentration of ionized Ca^{2+} in the plasma is greater than in the CSF.

In 1923, Barrio (24) found the Mg concentration in CSF in the range of from 1.15 to 5.02 mEq L^{-1}. The Mg content in CSF averages approximately 25% more than the

---

*1 mg Ca^{2+}/dL or mg Ca^{2+}% = 0.5 mmol $Ca_{1/2}^{2+}\ L^{-1}$ or mEq L^{-1}.

concentration in serum. Other researchers reported the range of Mg concentration to be 0.83 - 2.90 mEq L^{-1} with an average of 2.49 mEq L^{-1} (14) while Flexner (19) gave the range as 2.49 - 2.99 mEq L^{-1}. Atomic absorption and Titan yellow methods gave ranges from 2.0 - 2.7 mEq L^{-1} and from 2.2 to 3.0 mEq L^{-1} in CSF (23). The mean Mg concentration in serum of normal males was reported as 1.95 ± 0.21 mEq L^{-1} while the mean CSF value was 2.40 ± 0.14 mEq L^{-1} (15).* Although CSF has a higher concentration of Mg than serum, it contains less of the ultrafilterable fraction (25,26).

The higher concentration of Mg in CSF can be explained by the fact that it is secreted into the spinal fluid by cells of the choroid plexus and maintained at that level by the blood-brain barrier. This hypothesis was proven in 1927 by Cohen (27) who compared the Mg concentration of patients with and without meningitis. In the latter case, Mg concentration in CSF exceeded values for plasma by 0.39 - 1.40 mEq L^{-1}, while for those with meningitis the CSF Mg never exceeded plasma Mg by more than 0.09 mEq L^{-1} (in some cases, plasma had higher Mg concentration).

Plum in 1961 (28) could not find any significant

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*1.0 mmol $Mg^{2+}_{1/2}$ L^{-1} = 1.0 mEq Mg^{2+} L^{-1} = 0.50 mmol $Mg^{2+}L^{-1}$

difference in the concentration of CSF Mg between normal group and patients with multiple sclerosis. On the other hand, the ratio of Mg content of CSF and serum is fairly constant being on the average 1.26, while in multiple sclerosis it is about 0.93.

In the case of phosphorus, its metablism is closely linked to that of calcium. Phosphorus is one of the most important constituents of the central nervous system tissues. It occurs as inorganic or organic phosphate in blood. The normal range for inorganic phosphorus in CSF is 0.9 - 2.0 mg % (29). Some authors (30, 31) maintain that the CSF concentration of inorganic phosphorus is only 50% of the serum concentration, while others believe that the cerebrospinal fluid contains only 40% of the phosphorus concentration in the blood.

4. Blood-brain and Blood-CSF Barriers

The difference in concentrations of various substances in the CSF as compared to the serum points to the fact that a blood-brain barrier exists (which cannot be explained by mere diffusion). This barrier forms soon after birth.

For instance, in erythroblastosis fetalis (a disease in which infants are severely jaundiced), the cerebral capillaries of infants are more permeable than those of the adults (3), thereby enabling bile pigments to penetrate the central

nervous system. However, severe jaundice in adults does not affect neural function, because the blood-brain barrier prevents the passage of bile pigments into the brain.

The blood-brain barrier is permeable to water, oxygen and carbon dioxide. Glucose can be transported with relative ease into the neural tissue whereas chloride, bicarbonate, magnesium, sodium, potassium and phosphate in plasma require three to thirty times longer to reach equilibrium with CSF (3). The blood-brain barrier forms no obstacle to small amounts of catecholamines and proteins though it slows down the movement of urea into the CSF. The barrier is completely permeable to some drugs like sulfadiazine and erythromycin while only partially permeable to chlortetracycline and penicillin (3).

The blood-brain barrier helps to maintain, within precise qualitative and quantitative limits, the composition of the cerebrospinal fluid. In regions of the brain where infection, irradiation and tumors occur, the blood-brain barrier is destroyed.

In normal cases, a pH difference of 0.1 unit exists between blood and brain. Generally, substances with a high lipid solubility (higher partition coefficient) tend to cross the barrier more quickly than do others with a lower lipid solubility.

The blood-cerebrospinal fluid barrier is due to the high functional impermeability of the choroid epithelial cells to certain substances. In order to enter the CSF, the substance must initially go through the capillary endothelium and then the epithelial cells of the choroid plexus (1-3). Metabolites and drugs leave the CSF via the arachnoid villi, which occur rapidly, regardless of molecular size or lipid solubility. Large protein molecules, like plasma albumin, exit quickly via this route. The rates of exit for all substances are the same and depend on the rate of bulk flow of CSF through the arachnoid villi. On the other hand, some drugs and lipid-soluble metabolites can leave the brain by passive transport across the barrier from the extracellular fluid.

5. Pathology

The normal CSF is a clear fluid resembling water (32). Turbidity suggests an increase in white and red blood cells, as well as protein. This may be indicative of infections. If the CSF is purulent, then purulent meningitis and menigoencephalitis may be suspected. A thorough examination of CSF and, in particular, the determination of calcium, magnesium and phosphate may be important in the differential diagnosis of certain diseases.

Calcium and phosphate levels are regulated by the

parathyroid gland and their metabolisms are closely related (23). More than 99% of Ca and 80% of P are present in the bones as calcium fluorophosphate apatite, while the rest of Ca and P have different functions. For instance, calcium ions participate in blood coagulation, decrease neuromuscular excitability and activate certain enzymes like adenosine triphosphatase and succinate dehydrogenase. They may also be involved in the release of neurotransmitters and in the transfer of inorganic ions across cell membranes.

On the other hand, phosphate is involved in the intermediary metabolism of carbohydrates and the mineralization of bone, besides being a component of important molecules like nucleic acids, nucleotides, phospholipids and organic phosphate esters (23, 33). Excellent review articles on the metabolism and function of Ca and P in biological systems have been written in the past decade (33-38).

In the case of magnesium, it is the second most abundant intracellular cation after potassium (32,39). Magnesium acts as an enzyme activator for a number of physiologically important enzymes like creatine kinase, alkaline phosphatase, adenosine triphosphatase, and hexokinase which are involved in transfer and hydrolysis of phosphate groups. Several reviews of Mg metabolism and biochemistry have been published (39-47).

It is beyond the scope of this thesis to discuss in

detail the biochemistry, metabolism, functions and requirements of calcium, magnesium and phosphorus. However, some of the most important aspects of these ions have already been mentioned. The reader is directed to previously cited references (33-47). A partial list of diseases commonly found with high and low levels of these ions is given in Table II. It can be seen that the diagnosis of numerous diseases depend on the accurate and precise analytical determinations of these ions which will be dealt with in more detail in the next section. The necessity for simple, sensitive, specific, precise, efficient and rapid techniques is obvious as only slight variations from the normal levels indicate the presence of disease.

In clinical neurology, examination of CSF is a routine procedure, as the composition and the pressure of this fluid is an invaluable aid to diagnosis. The samples are normally withdrawn by a long spinal needle that taps the large subarachnoid space beneath the level of the spinal cord, between the 4th and 5th lumbar vertebrae. This procedure is generally known as a lumbar puncture or spinal tap (2,32).

B. ANALYTICAL METHODS

1. Review of Various Techniques

Various analytical methods are available for the determination of Ca, Mg and P in biological fluids. In general

TABLE II

DISEASES CONNECTED WITH HIGH AND LOW LEVELS OF CALCIUM, MAGNESIUM AND PHOSPHORUS[a]

Hypercalcemia	Hypocalcemia	Hyperphosphatemia	Hypophosphatemia	Hypermagnesemia	Hypomagnesemia
Hyperparathyroidism with hypophosphatemia	Hypoparathyroidism	Hypoparathyroidism	Hyperparathyroidism	Addison's disease	Hypoparathyroidism
Paget's disease with increased alkaline phosphatase	Osteomalacia	Chronic glomerular disease with high creatinine	Childhood rickets, adult osteomalacia	Diabetic acidosis	Acute pancreatitis
Thyrotoxicosis	Pregnancy	Sarcoidosis	Chronic use of antacids with aluminium hydroxide	Dehydration	Aldosteronism
Malignant tumors with or without bone metastasis	Pancreatitis	Milk alkali syndrome	Drastic correction of hyperglycemia and diabetic ketoacidosis	Uremia	Chronic alcoholism and delirium tremens
Acute bone atrophy	Nephrosis, nephritis	Hyperthyroidism	Renal tubular acidosis, De Fanconi syndrome		Digitalis intoxication
Hypervitaminosis D	Respiratory alkalosis and hyperventilation	Hypervitaminosis D	Malabsorption syndrome and hyperinsulinism		Chronic glomerulonephritis
Polycythemia vera	Diuretic intake	Pseudohypoparathyroidism			Malabsorption syndrome
Acromegaly	Hypomagnesemia secondary to suppression of release of parathyroid hormone	Malignant hyperpyrexia			
Cushing's syndrome	Chronic steatorrhea	Increased growth hormone secretion			
Multiple myeloma					
With hypergamma-globulinemia 1) sarcoidosis 2) malignancies					

[a] The contents of this table are adapted from Henry (16) and Tietz (23).

atomic absorption spectrometry is regarded as the reference methods for most inorganic ions.

Traditional analytical techniques for calcium and magnesium are tedious and time consuming. However, complexometric titration gives an efficient and rapid determination which has been widely applied (48).

Table III gives a list of different methods available for calcium, magnesium and phosphate determinations. As can be seen from the table, a wide variety of techniques exist for the analyses of these ions. However, the spectrophotometric methods will be dealt with in more detail. Table IV is a list of the more important reagents used for calcium and magnesium determinations employing visible spectrophotometry. Though there are numerous reagents available for both these ions, some of the colorimetric procedures are complicated and lack sensitivity.

In 1956, Zak et al. (49) used Eriochrome Black T and ethylenediaminetetraacetic acid (EDTA) in the titration of both calcium and magnesium in spinal fluid. The technique is tedious requiring centrifugation and a large volume of sample (2 mL). Thus this method would not be suitable for emergency cases.

With regard to the determination of phosphorus, the ammonium phosphomolybdate technique is widely used. Numerous

TABLE III

ANALYTICAL METHODS FOR CALCIUM, MAGNESIUM AND INORGANIC PHOSPHATE[a]

Calcium	Magnesium	Phosphate
Atomic absorption	Atomic absorption	Photometric determination of ammonium phosphomolybdate
Flame photometry	Flame photometry	
Ion-selective electrode	Fluorescence spectrophotometry	Indirect ultramicro phosphorus determination by 2-octanol extraction and atomic absorption of Mo
X-ray fluorescence	Precipitation of $MgNH_4PO_4$ and determination of phosphate	
Neutron activation		Photometric determination of molybdivanadophosphoric acid
Oxalate titration	Precipitation of Mg 8-hydroxyquinoline or the fluorescence of this compound	
Chelation with EDTA		Photometric determination with malachite green
Precipitation by chloranilic acid or naphthylhydroxamic acid and photometry	Spectrophotometry of dye-lake indicators and other reagents	Enzymatic method and fluorescence determination
	EDTA titration using a variety of complexometric titrations	
Spectrophotometric methods with various indicators	Special techniques like ion exchange, emission spectrography, X-ray spectroscopy and polarography	

[a]The contents of this table are adapted from Henry (16) and Tietz (23).

TABLE IV

REAGENTS FOR DETERMINATION OF CALCIUM AND MAGNESIUM[a]

Calcium	Magnesium
Murexide	Magnesium ammonium phosphate precipitate methods
Calcon, Cal-Red	Ferric thiocyanate
Eriochrome blue-black, acid chrome blue-black	8-Hydroxyquinoline
Omega chrome blue-green	Ceric sulphate
Laquer scarlet C	Titan yellow
Calmagite	EDTA-Eriochrome Black T
Thymolphthalexone	Methylthymol blue
Calcein	Calmagite
Glyoxal-bis-(2-hydroxyacid)	Magon, magon sulfonate
Chromazurol S, calichrome	
Pyrogallol-carboxylic acid, pyrocatechol violet	
Acid alizarin black	
Methylthymol blue	
Cresolphthalein complexone	

[a]The contents of this table are adapted from Henry (16) and Tietz (23).

reducing agents have been tried in the past and they are listed in Table V. The reagents and methods chosen for the present study will be discussed in greater detail in the following sections.

2. Reagents for Calcium and Magnesium Determinations

(a) Cresolphthalein Complexone

Cresolphthalein complexone was first used in 1954 (50, 51) for the detection of calcium, strontium and barium in trace quantities and had been adapted for use in clinical determinations (50,52-54). In one indirect method, calcium was precipitated as oxalate (55) before reacting with CPC. Another technique used dialysis for the removal of protein and cyanide for stabilizing the base reagent (56).

Magnesium interference was removed with the addition of 8-hydroxyquinoline (57) which incidentally reduced the molar extinction coefficient of calcium with CPC from 2.1×10^4 to 1.4×10^4 mol cm^{-2} (52). An automated procedure with the incorporation of 8-hydroxyquinoline was introduced in 1967 (58).

Sarkar and Chauhan (52) used an ammonia/ammonium chloride buffer (pH 10.5) in the system, but the procedure lacks reproducibility due to the presence of a high and variable absorbance blank (53). Dimethylsulfoxide (DMSO)

TABLE V

REDUCING AGENTS FOR INORGANIC PHOSPHATE DETERMINATION[a]

Various reducing agents	
Stannous chloride	Aminonaptholsulfonic acid
Phenylhydrazine	p-Methylaminophenol sulfate (Elon)
Hydroquinone	2,4-Diaminophenol·HCl
Ferrous sulfate	Hydrazine sulfate
Ascorbic acid	N-Phenyl-p-phenylenediamine

[a]The contents of this table are adapted from Henry (16).

was incorporated (53) into the reagent to increase solubility of CPC and 8-hydroxyquinoline, and also to stabilize the system with reduction of the blank absorbance.

Under acidic conditions, CPC is unstable in aqueous solution (59) but it has been shown that diethanolamine (DEA) improves the stability of the colour reagent at a high concentration of CPC. Absorbance increases with increasing pH to a certain optimum pH. No interference from jaundiced, hemolytic or moderately lipemic specimens is observed (53,60)

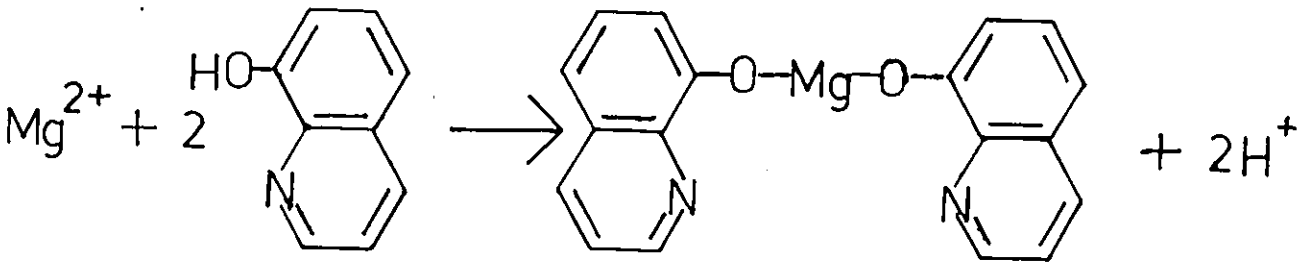
One advantage of CPC over other reagents is that phosphate does not interfere (52) up to a concentration of 12 times greater than that of calcium, while phosphate causes considerable interference in flame photometric and atomic absorption methods (61,62). Linearity is observed up to a concentration of 1.6 ppm Ca with CPC (63).

The addition of methyl alcohol stablizes the reagent (64) and linearity is extended to 25 mg/dL of Ca. Cohen and Sideman (65) modified the Moorehead and Briggs method (66) for determination of calcium in serum. In a recent article, Lorentz (63) recommended the addition of 5 M urea to the system to enhance the absorption of the Ca-complex. In order to prevent interference from copper, cobalt, nickel and iron, potassium cyanide can be added to the DEA buffer (48,53) to chelate these ions. The accepted standard

deviation range for calcium is from 0.07 - 0.40 while the mean coefficient of variation is 2.3% for analytical methods (67).

The structure of the CPC molecule is shown in Figure 2.

CPC has a maximum absorbance at 575 nm when calcium is complexed with it. The intensity of the violet-pink colour is proportional to the concentration of calcium. The original yellow blank turns a light violet-pink on alkalinization with DEA buffer. As 8-hydroxyquinoline is dissolved in the colour reagent, it has to be added before the buffer in order to permit the 8-hydroxyquinoline to complex with any Mg ions present. The proposed reaction between Mg and 8-hydroxyquinoline is believed to be the following (68):



8-Hydroxyquinoline Mg - 8-Hydroxyquinoline Complex

FIGURE 2

STRUCTURES OF REAGENTS FOR
CALCIUM AND MAGNESIUM

Legend

The structures of CPC, Calmagite, MTB and Magon are shown.

A: Cresolphthalein complexone ($C_{32}H_{32}N_2O_{12}$) Chemical name is cresolphthalein 3',3"-bis-methylene-iminodiacetic acid

M.W. 636.62 λ_{max} complex 575 nm

B: Calmagite ($C_{17}H_{14}N_2O_5S$) Chemical name is

[1-(1-hydroxy-4-methyl-2-phenylazo)-2-naphthol-4-sulfonic acid]

M.W. 360.39 λ_{max} complex 525 nm

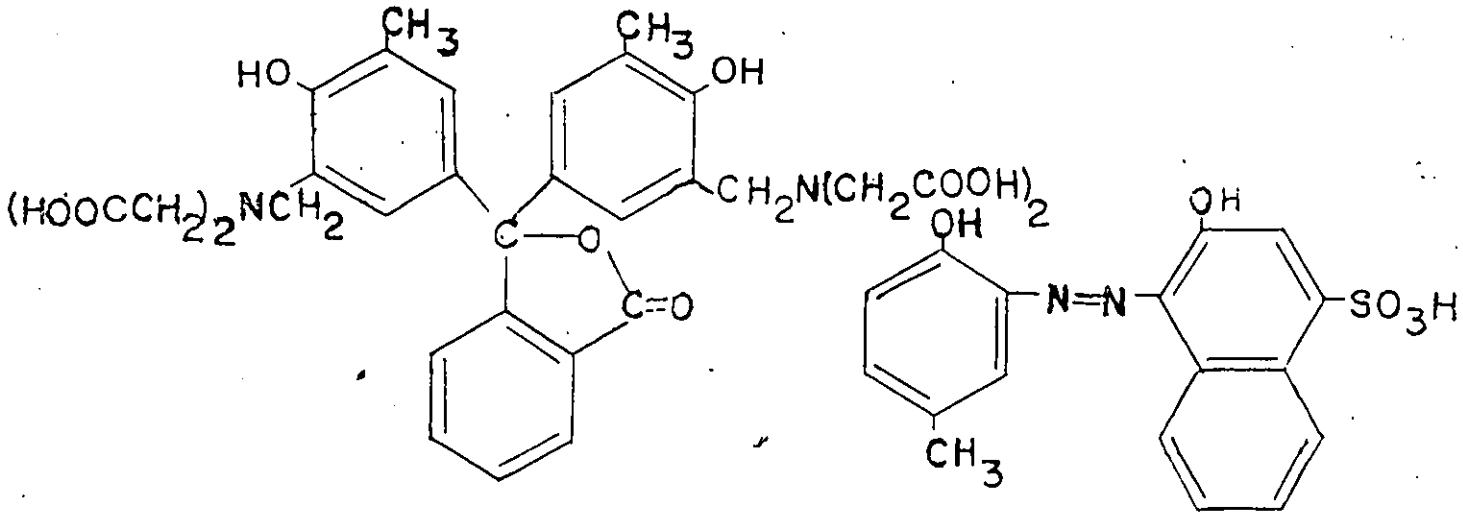
C: Methylthymol Blue, sodium salt ($C_{37}H_{46}N_2O_{13}SNa$) Chemical name is 3,3'-bis {[N',N-di(carboxymethyl) aminol]-methyl }-thymolsulfonephthalein pentasodium salt

M.W. 844.76 λ_{max} complex 610 nm

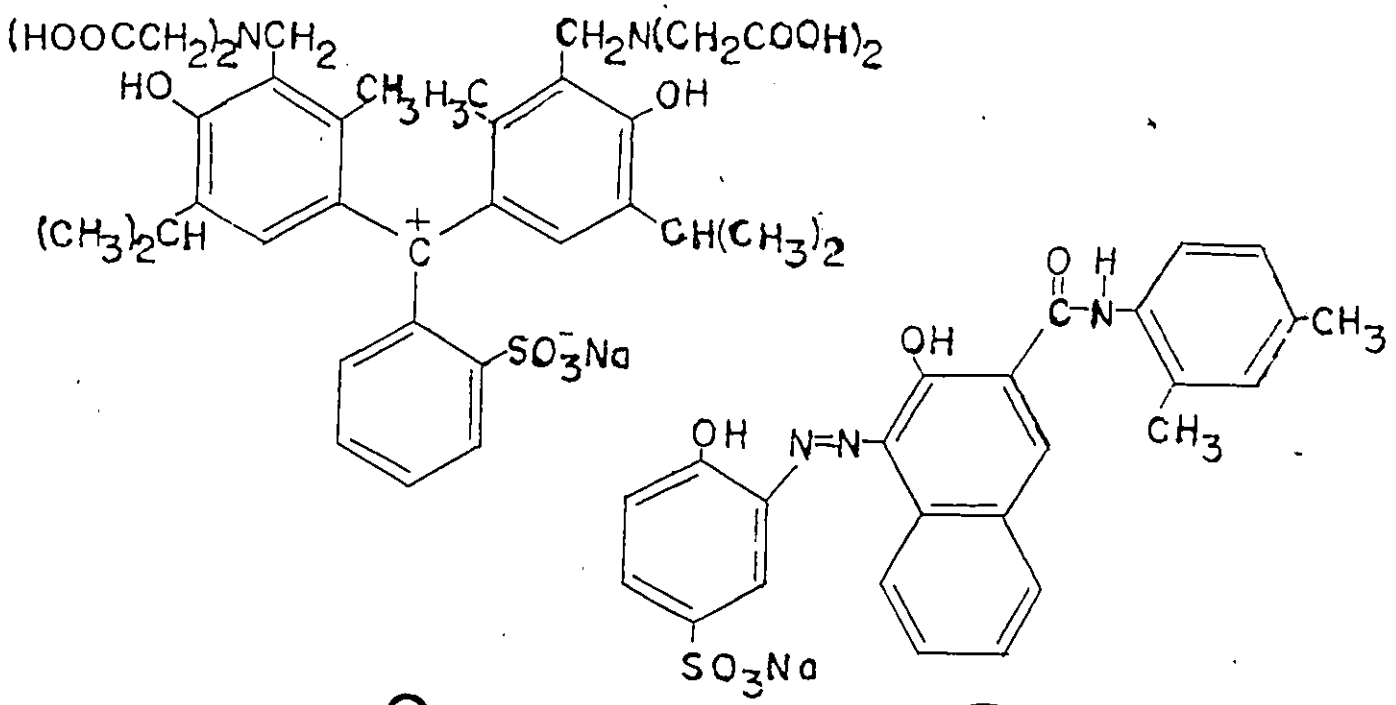
D: Magon sulfonate (Xylidyl Blue I) ($C_{25}H_{20}N_3NaO_6S$) Chemical name is sodium 1-azo-2-hydroxy-3-(2,4-dimethyl carboxanilido)-naphthalene-1'-2(hydroxy-benzene-5-sulfonate).

M.W. 513.51 λ_{max} complex 548 nm

FIGURE 2



A B



C D

(b) Calmagite

Calmagite was first introduced by Lindstrom and Diehl in 1960 (69) as an indicator for the determination of calcium and magnesium. It belongs to the Eriochrome dye family but differs from them by its greater stability in aqueous solution (70). A commercial kit for Mg determination, patented by Pierce Chemical Co., Rockford, IL, 61105 (71) uses the procedure of Gindler and Heth (72). The method has also been automated (73). Weil (74) used Calmagite to determine calcium at pH 10-10.6.

Recently, other researchers added a detergent, Empigen BB, to improve the system (75). The detergent causes an increased separation of the test and blank spectral absorbance bands. The blank peaks show a bathochromic shift. Chauhan and Sarkar (76) used 0.04% of Calmagite and a potassium chloride/sodium hydroxide buffer (pH 12.0) in their determination of Mg. This is a more aqueous system than the modified method containing DMSO used in the present study. The linearity in Gindler and Heth's (72) method is only to 2 mmol L^{-1}, measured at λ_{max} 532 nm.

While cyanide was used in the present study to mask iron and other trace metals, other researchers resorted to triethanolamine (75). Calcium, copper, iron and zinc give positive interference, while manganese and oxalate show a

suppressant effect (76). The formation constants of Calmagite-Mg and calcium complexes are quite high (log K_{MgI} = 8.1, log K_{CaI} = 6.1). Because the ratio of the stability constants is 100:1, calcium will interfere in Mg determinations (70).

EGTA is an ideal masking agent for calcium - the logarithm of the stability constants of the calcium and magnesium complexes are log K_{CaX} = 11.0 and log K_{MgX} = 5.2, respectively (70). Calcium would be chelated in preference to Mg because the log of its stability constant is almost twice that of Mg. Calcium interference in Mg determinations can be removed if EGTA is used as masking agent even if the calcium concentration is ten times that of magnesium (76) and linearity holds till 0.5 ppm of Mg. The optimum pH is reported as 12.0 while higher pH's lead to precipitation of magnesium hydroxide.

The maximum peaks for the Calmagite blank and Calmagite-Mg complex are at 560 and 525 nm, respectively. The molar extinction coefficients of various Mg complexes are shown in Table VI.

The reagent blank is deep blue in an alkaline medium and becomes reddish on addition of Mg. The advantages of this reagent are: its stability at room temperature for a week; colour formation is complete within 15 min; absorbance remains constant for four hours; no protein

TABLE VI

MOLAR ABSORPTIVITY OF VARIOUS MAGNESIUM COMPLEXES[a]

Indicator	Molar absorptivity mol cm^{-2}
8-Hydroxyquinoline	3.43×10^3
CPC	9.60×10^3
MTB	1.52×10^4
Calmagite	1.74×10^4
Magon sulfonate	3.04×10^4
Magon[b]	4.30×10^4

[a]Reference (76).

[b]Molar absorptivity varies with differing concentrations. Under ethanolic conditions (pH 10.90-11.60) the final solution has λ_{max} 620 nm.

precipitation is necessary; and results are unaffected by citrate in serum or plasma (71).

The structure of Calmagite is illustrated in Figure 2.

(c) Methylthymol Blue

Korbl and Pribil (77) were the first in 1957 to describe methylthymol blue (MTB) as a useful indicator for many metals including magnesium. Metcalfe in 1965 (78) determined Mg with MTB (λ_{max} 610 nm) in a medium buffered with ammonia/ammonium chloride solution. Maximum colour formation occurs around pH 10.4 - 11.3 and thus the buffer is adjusted to pH 10.8

In an ammonia/ammonium chloride buffer system at pH 10, MTB gives good blue-to-colourless (or faint grey) end points in the titration of cadmium, cobalt, lead, magnesium, manganese or zinc (77,79,80). However, in a sodium hydroxide medium at pH 12, good blue-to-colourless end points are obtained in the titration of the three alkaline earth metals (80). When the pH goes above 12.5, a blue ionization form of the free indicator is obtained.

Gindler and King (81) determined calcium (λ_{max} 612 nm) at pH 11.5, by incorporating an aqueous system of two solutions, namely MTB with 8-hydroxyquinoline, polyvinylpyrrolidone and hydrochloric acid as one solution, and monoethanolamine plus sodium sulfite as the buffer. A blue colour forms

immediately, which is stable for several hours. The molar absorptivity of the calcium complex is 1.29×10^4 mol cm^{-2} (81) while that of the magnesium complex is 1.52×10^4 mol cm^{-2} (78).

In a direct determination of Mg in serum, Connerty et al. (82) reported a linear range of 0-8.0 mg dL^{-1} measured at 600 nm, and a colour stability of 30 minutes. The structure of MTB is shown in Figure 2.

(d) Magon

In 1956, Mann and Yoe (83) were the first to use Magon sulfonate (Xylidyl Blue I) in the determination of magnesium. Synthesis of the non-sulfonated compound, Magon (Xylidyl Blue II), was carried out the following year (84) for the same purpose. Both are red solids; though the former is soluble both in water and alcohol, the latter is essentially insoluble in water but soluble in organic liquids.

The technique, as described by the original researchers (83,84) had many disadvantages, like slow reaction times and interferences from calcium (85). Several investigators applied the technique to the determination of magnesium in biological fluids but using different amounts of ethanol as the solvent and borax as the buffer (86-89) at various pH's. Vanden Bossche and Wieme (89) in 1966 adapted the system for automation. They found that phosphate would not interfere at

strongly alkaline pH if the magnesium was first complexed with the colour reagent at neutral pH.

Svoboda and Chromy (85) recommended the use of a borax buffer at pH 9.50 and a 50-60% (v/v) ethanol concentration as the optimum conditions. They measured absorbances around 505 and 620 nm and found that phosphate at high concentrations (>10 x Mg) interfered by decreasing the absorbance at 505 nm and increasing the absorbance at 620 nm.

In the modified method by Baginski et al. (90), DMSO replaces ethanol and potassium hydroxide replaces borax, while calcium interference is eliminated by incorporating EGTA in the system. Other trace metals are masked by cyanide. Pragay et al. (91) evaluated a commercial kit by E. M. Diagnostics, Darmstadt, West Germany, utilizing Magon in an alcoholic solution with a reducing agent. The magnesium complex has a maximum absorbance at 505 nm with a borax buffer. They found that the technique required only 20 µL of sample, needed no deproteinization and was more sensitive than the Titan yellow method. The colour is stable for 30 minutes and as it is simple and rapid, the kit is suitable for stat cases. However, hemolysis, lipemia and high bilirubin content interferes with the procedure (91), while calcium (up to 14 mg %) does not show any significant interference.

In another study, citrate was found to have a negative

interference with the reaction (92). Thus citrated plasma specimens, or specimens from patients who have transfusions cannot be used. However, determination of magnesium in CSF presents no problem. Moreover, linearity is observed up to 0.4 ppm (83) and the optimum precision range is 0.12 - 0.4 ppm of magnesium. The reaction is not sensitive to temperature changes.

The properties of both compounds are somewhat similar with a few exceptions. Magon sulfonate is an acid-base indicator with a transition from red to blue-violet at pH 7-8 and another from blue-violet to pink at pH 11-12. In an aqueous medium, magnesium causes a change from blue-violet to red in the pH range of 8-11 (83) but in an alcoholic medium, the change is from blue to salmon-pink.

Similarly, an acidic ethanolic solution of Magon is red, while a neutral or weakly basic solution is blue and in a strongly basic solution the colour is red. In ethanolic solution, magnesium also causes a change from blue to salmon pink. Under these conditions, the reagent shows a maximum peak at 620 nm, while the complex has 2 maxima, namely, 505 and 535 nm (84). However, when DMSO is used as the solvent and potassium hydroxide as the base (90), the complex has two maximum absorbance peaks which occur at 510 and 548 nm Figure 2 shows the structure of Magon sulfonate.

In the present study, the method by Baginski et al. (90) was used because of the simplicity of the procedure and the removal of a deproteinization step. DMSO was a better solvent than ethanol in stabilizing the system. The technique is very sensitive as only .10 µL of sample is required.

3. Methods for Phosphate Determinations

There are several methods available for the determination of inorganic phosphate in biological fluids. Fawaz and Tejerian used an enzymatic method for their determination (93). Other researchers employed the phosphomolybdate systems (94,95), malachite green method (96) and even a centrifugal analyzer (97). Some of the methods are tedious and complicated or require expensive instrumentation.

Several modifications have been made to the Fiske and Subbarow technique (98). Baginski and Zak (99) in 1960 introduced citrate-arsenite reagent to the phosphomolybdate system in the determination of phosphate in serum. Arsenite increases the sensitivity of the reaction while citrate complexes excess molybdate and prevents it from reacting with any inorganic phosphate released by hydrolysis (100).

Incorporation of DMSO into the arsenite-citrate reagent seems to enhance the molar absorptivity, and probably has a clearing effect on the system (99,101,102). Detergents

have been used by several investigators (94,95,103),to counter turbidity. Baginski et al. (100) introduced sodium dodecyl sulfate (SDS) into the system for the same purpose.

Sodium dodecyl sulfate keeps the protein in dispersed form while ascorbic acid acts as the reducing agent. The 'heteropoly' blue complex exhibits two absorbance maxima, namely, 700 and 840 nm (100). Absorbances are measured at the lower wavelength as Beer's law is obeyed up to a value of 1.05 (99). The colour is stable for several hours. Hemoglobin and bilirubin do not interfere at these wavelengths as the absorbance peaks of these compounds are different from the 'heteropoly' blue complex.

The principle of the reaction may be considered as (100):

$$12\ MoO_4^{2-} + H_3PO_4 + 24\ H^+ \longrightarrow H_3PMo_{12}O_{40} + 12H_2O$$

Molybdate is changed into a heteropolyacid in an acid environment, which then reacts with phosphate to form a complex. On reduction, the acid forms a blue product, known as 'heteropoly blue.'

The reaction between phosphate and molybdate is not instantaneous; a minimum of 2 min is needed for completion of reaction before the addition of arsenite-citrate

reagent. When the time between the addition of molybdate and citrate is extended, a blue colour forms which does not correspond to the concentration of phosphate but to some other reaction of molybdate (99). However, when citrate reagent is added, the colour decreases gradually till the intensity reflects the actual concentration of phosphomolybdate.

All reagents are stable at room temperature for an indefinite period except ascorbic-SDS solution which is only stable for two months. The method of Baginski et al. (100) is followed in the present study with a slight modification. Glacial acetic acid is added to the ammonium molybdate reagent (102).

C. THE STUDY

The purpose of this project, is to find the most sensitive and specific reagents for the determination of calcium, magnesium and phosphate, respectively, in cerebrospinal fluid, employing spectrophotometric techniques. Sensitivity is of the utmost importance in order to detect minute levels of analytes coupled with µL volumes of samples. This factor becomes a major priority especially in cases of pediatrics specimens.

Specificity is another factor to be considered in evaluating the reagents, as each reagent has to be specific

for an individual ion without any interference from the other ions that are present. Thus, an ideal reagent would allow one to determine the concentration of the analyte accurately and precisely at the μmol level in microliter samples and simultaneously be specific for only that ion. This would eliminate the need for several milliliters of cerebrospinal fluid for analyses. The necessity for minute quantities of specimens cannot be overemphasized.

Another important aspect to bear in mind is the speed and relative ease of a determination. The method chosen must be simple with a rapid turnover time in order to be applicable in a clinical laboratory, otherwise it would only be of a purely academic value. One has also to take into consideration the economic feasibility of running numerous analyses each day in the clinical laboratory.

The ideal reagent should be inexpensive and fairly stable at room temperature for several months and the procedure should ideally be adaptable to automation if the need arises. The technique best suited for the clinical laboratory must be one that does not require expensive equipment, large turnover time or complicated procedures. Though atomic absorption is regarded as the reference method for most inorganic cations, small clinical laboratories or hospitals may not have the facilities to do the analyses.

However, most hospitals would have a spectrophotometer and thus spectrophotometry would be the method of choice for practical purposes.

With these factors in mind, various reagents for the determination of Ca, Mg and P in CSF were studied. The reagents employed for calcium determination were CPC, Calmagite and MTB, whereas those for magnesium determination were Magon, Calmagite and MTB. For the determination of inorganic phosphate, the phosphomolybdate method was investigated for direct application in CSF.

In the case of calcium and magnesium determinations, respectively, three reagents were studied under similar conditions for each ion. The preliminary studies examined the analytical aspects such as buffer and solvent systems, positive or negative interferences from other ions, reagent specificity and sensitivity by comparison with each other.

The second part of the investigation dealt with the application of these methods in clinical studies. Pooled CSF was used for these purposes. Recovery studies, within-run and day-to-day precision studies were performed on the clinical specimens for a thorough investigation of each method.

CHAPTER II

EXPERIMENTAL

A. ANALYTICAL STUDIES

1. Equipment

Balance: A Mettler PC 4400 Delta Range supplied by Fisher Scientific Co., Don Mills, Toronto, Ontario M3A 1A9 was used for values above one gram and a Mettler, Type H16 balance from the same company was used for weights less than one gram.

Computer: University of Windsor's WILBUR terminal was used to access the central terminal's SAS programs for statistical analysis (regression, C. V. and S. D.).

Glassware: Borosilicate test tubes (13 x 100 mm) Pyrex beakers, volumetric flasks and graduates glass pipettes were used throughout the studies. Volumetric pipettes were used for measuring volumes greater than one milliliter.

Micropipettors: An Oxford pipettor, 200 μL, from Canadian Laboratory Supplies, Ltd., Toronto, Ontario, M8Z 2H4 and Gilson Pipetman Models P-200D and P-1000D with disposable pipette tips C20 and C200 available from Mandel Scientific Co. Ltd., 395 Norman St., Ville St.

Pierre, P.Q. H8R 1A3, were used in the studies.

pH Meter: An Accumet Model 120 from Fisher Scientific Co., and a Corning Digital 111 pH meter from Canadian Laboratory Supplies Ltd., Toronto, Ontario, M8Z 2H4 were used. Electrodes were supplied by Graphic Controls, Recording Chart Division, Buffalo, NY, 14240.

Spectrophotometer: Beckman ACTA MVI spectrophotometer available from Beckman Instruments, Inc., Scientific Division, 901 Oxford St., Toronto, Ontario, M8Z 5T2 was used to measure absorbances.

Vortex: A Vortex-Genie™ available from Scientific Industries, Inc., Bohemia, NY, 11716, was used for thorough mixing of solutions in test tubes.

2. Materials

Water: Deionized distilled water purified by a Barnstead column from Barnstead, Boston, MA , 02132 was used throughout studies.

Chemicals:

Ascorbic acid, diethylamine (DEA) and dimethyl sulfoxide (DMSO) were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI , 53233 (ACS grade).

Ammonium Molybdate was purchased from Anachemia Chemicals Ltd., Mississauga, Ont., L5C 1T7, while glacial acetic acid, potassium hydroxide and potassium dihydrogen phosphate were purchased from BDH Chemicals, Toronto, Ontario, M8Z 1K5.

Cresolphthalein complexone and Magon (Xylidyl Blue I) were supplied by J. T. Baker Chemical Co., Phillipsburg, NJ, 08865.

Calmagite was purchased from G. Frederick Smith Chemical Co., Columbus, OH, while both ethyleneglycol bis-(2-amino-ethyl)-NNN'N' tetraacetic acid (EGTA) and Methylthymol blue (MTB) were obtained from Sigma Chemical Co., St. Louis, MO 63178.

The following compounds were purchased from Fisher Scientific Co., Fair Lawn, NJ, 07410: ammonia; ammonium chloride; DMSO; 8-hydroxyquinoline; hydrochloric acid; potassium cyanide; potassium hydroxide; potassium chloride; sodium arsenite; sodium citrate; sodium metabisulfite; sulfuric acid; urea; and atomic absorption stock standards of calcium and magnesium.

3. Reagents

(a) General

Calcium Standards: All aqueous calcium standards ranging from 0.50 mM to 3.00 mM in concentration were prepared from an atomic absorption stock standard of 250.00 mM Ca^{2+} (10 mg mL^{-1}). They were then stored in polyethylene bottles.

Magnesium Standards: All aqueous magnesium standards ranging from 0.5 mM to 3.00 mM in concentration were prepared from an atomic absorption stock standard of

411.35 mM Mg^{2+} (10 mg mL^{-1}). They were also stored in polyethylene bottles.

Phosphate Standards: A stock phosphate standard of 1.0 mg mL^{-1} was prepared by dissolving 438.1 mg of potassium dihydrogen phosphate (KH_2PO_4) in deionized distilled water and diluting to 100 mL in a volumetric flask.

Phosphate working standards ranging in concentration of 1.0 mg dL^{-1} to 8.0 mg dL^{-1} were prepared from the stock standard and then stored in polyethylene bottles.

Mixtures of Calcium and Magnesium Standards for Interference Studies: Various aqueous mixtures of Ca and Mg standards ranging from low to high concentrations were mixed in different proportions as given in Table VII.

(b) Reagents for Calcium Determination

(i) CPC - (0.0628 mM)

Without Urea. The colour reagent was prepared according to the paper of Baginski et al. (53) by dissolving 0.040 g of cresolphthalein complexone in a beaker containing 1.0 mL of concentrated hydrochloric acid with constant stirring. A few drops of water helped in the wetting of the particles. This was then followed with the addition of 1 dL of dimethyl sulfoxide (DMSO) and 2.5 g of 8-hydroxyquinoline (8-HQ) in a 1-L volumetric flask. The yellow reagent was thoroughly mixed and diluted to volume with distilled water before transferring to a polyethylene bottle.

TABLE VII

CALCIUM AND MAGNESIUM STANDARDS
FOR INTERFERENCE STUDIES

	Mg^{2+} mM	Ca^{2+} mM
A	0.856	0.950
B	0.856	1.125
C	0.856	1.300
D	1.250	0.950
E	1.250	1.125
F	1.250	1.300
G	1.645	0.950
H	1.645	1.125
I	1.645	1.300

Diethylamine buffer (DEA): To a 1-L volumetric flask, 0.5 g of potassium cyanide (KCN) and 40.0 mL of DEA were added and subsequently diluted to volume with water. The colourless solution was then stored in polyethylene bottle.

With Urea (5M). The CPC reagent was prepared as described in the above paragraph with a slight modification. Lorentz in 1982 (63) reported the increase in absorption by the addition of 5M of urea to CPC. Thus, 300.2 g of urea crystals were added to the reagent and made up to 1-L with constant stirring, though ethanol was not added to this as given in the original paper (63). The reason was to see what effect the addition of urea alone had on the original system keeping everything else constant. If colour absorption was enhanced, then this could be attributed to the presence of urea in the system and not to the change of solvents. The reagent was also stored in a polyethylene bottle.

Two DEA buffers were made as previously described, but one contained 5 M urea. The pH of the buffer without urea was found to be 11.95 while the other with urea was adjusted to pH 11.7 by glacial acetic acid (63).

(ii) Calmagite (1.109 mM). This reagent was made by dissolving 0.40 g Calmagite and 2.5 g of 8-hydroxyquinoline in 1 dL of DMSO and diluted to 1L with water, according to the CPC procedure (53). The only difference was the con-

centration of Calmagite (40% or 1.109 mM) as given by Chauhan and Sarkar (76) as the optimum concentration. The dark red reagent was then stored in a polyethyline bottle.

The DEA buffer was made up as described for CPC procedure.

(iii) MTB (0.0628 mM). The Methylthymol blue reagent was also made up according to (53) by dissolving 0.052 g MTB and 2.5 g of 8-hydroxyquinoline in 200 mL of DMSO and diluting to 1L with water after thorough mixing. Twice the amount of DMSO was added because 8-hydroxyquinoline settled out of solution when the original volume of DMSO was used (1dL was originally used).

The DEA buffer was made up as previously described.

(c) Reagents for Magnesium Determination

(i) Magon (0.1947 mM). The colour reagent was prepared according to the article by Baginski et al. (90) in 1982. Approximately 2 dL of DMSO were placed in a 500 mL beaker and 0.10 g of Magon sulfonate was added with constant stirring. In a 1-L volumetric flask, 0.100 g of EGTA was added followed by 2 pellets of potassium hydroxide to help in dissolving EGTA in about 800 mL of water. When all the EGTA had dissolved completely, the two solutions were thoroughly mixed with a magnetic stirrer and the final deep-blue solution was then stored in a polyethylene bottle.

Potassium hydroxide buffer (KOH pH 13.0). Approximately 16.0 g of potassium hydroxide pellets and 0.5 g of potassium cyanide (KCN) were dissolved in water and diluted to 1L before transferring to a polyethylene bottle.

(ii) Calmagite (1.109 mM). The Calmagite reagent was made up according to the Magon procedure (90) by dissolving 0.40 g Calmagite in 2 dL of DMSO and diluting with water to 1L. The red reagent was kept acidic at pH 3.7 because the reagent deteriorated rapidly and would not give as high an absorbance in an alkaline medium. The concentration of Calmagite was chosen to be 1.109 mM as this was the optimum concentration. (Thus EGTA was not dissolved in the colour reagent as originally described for Magon (90), as its dissolution would require an alkaline medium).

Potassium hydroxide buffer with EGTA (pH 12.00). The buffer was made up according to (90) with the extra addition of 0.10 g EGTA and diluting to 1L with water. The pH was adjusted to 12.0 (by adding a few drops of concentrated hydrochloric acid) as this was the optimum pH of Calmagite (76). The solution was then stored in a polyethylene bottle.

(iii) MTB (0.1947 mM). This reagent was prepared according to Chauhan and Sarkar (76) by dissolving 0.1644 g of MTB in 2 dL of DMSO and diluting it to 1L with water before transferring the brown-yellow liquid to a polyethylene bottle.

Ammonium chloride/ammonium hydroxide buffer with EGTA

(pH 10.2). This buffer was prepared according to Metcalfe (78) by dissolving 70 g of ammonium chloride in 568 mL of concentrated ammonium hydroxide solution (S.G. 0.900) with the extra addition of 0.10 g of EGTA and 0.5 g of potassium cyanide. The pH was kept at 10.0 to 10.8 and the solution was then stored in a polyethylene bottle. This buffer was used instead of the potassium hydroxide buffer because MTB gave higher absorbances with this medium.

(d) Reagents for Phosphate Determination

These were prepared according to the paper by Baginski et al. (100,102):

(i) Ascorbic acid-sodium dodecyl sulfate (A-SDS): Crysalline ascorbic acid (4.0 g) was dissolved in 100 mL of water. To this were added 0.5 g of sodium metabisulfite ($Na_2S_2O_5$) and 4.0 g of sodium dodecyl sulfate (sodium lauryl sulfate). The solution was mixed with a magnetic stirrer until everything had dissolved and transferred into an amber bottle. This was relatively stable at room temperature for two months and no refrigeration was required.

(ii) Dilute sulfuric acid: To a 1-L volumetric flask containing about 500 mL of water, 16.0 mL of concentrated sulfuric acid (H_2SO_4) was added and mixed thoroughly. After cooling to room temperature, the mixture was diluted to volume with water.

(iii) Ammonium molybdate in acetic acid (AMA): Ammonium molybdate tetrahydrate (5.0 g) was dissolved in 300 mL of water in a 1-L volumetric flask. Then 20.0 mL of glacial acetic acid was added and the mixture was diluted to volume with water and stored in a polyethylene bottle.

(iv) Arsenite-citrate in DMSO (ACD): Approximately 300 mL of water was added to dissolve 20.0 g of anhydrous sodium arsenite and 20.0 g of sodium citrate dihydrate in a 1-L volumetric flask. Dimethyl sulfoxide (400 mL DMSO) and 20 mL of glacial acetic acid were added to the solution before diluting it to 1 litre.

4. Methods

(a) Calcium Determination

(i) CPC: (Method of Baginski et al.) (53). The reagent blank was made up by pipetting 60 µL of water into a 13 x 10 mm borosilicate test-tube. Calcium standards (60 µL) of 0.5, 1.0, 1.5, 1.75, 2.0, 2.5, 3.0 mM were also pipetted in triplicate into other test-tubes. This was followed by 3 mL of colour reagent (CPC) and each tube was vortexed before the final addition of 3 mL of DEA buffer. They were then thoroughly mixed and allowed to stand at room temperature for 15 min. All the absorbances were read against a reagent blank. The spectrophotometer was set to zero with the reagent blank and read at 575 nm.

(ii) Calmagite: The above method (53) was followed except that only 1.5 mL of Calmagite reagent was pipetted before the final addition of 3 mL of DEA buffer. The absorbances were read at 525 nm.

(iii) MTB: For MTB reagent, the CPC procedure (53) was used with no change in volumes or the sequence of addition of reagents. The absorbances were read at 610 nm.

(b) Magnesium Determination (Method of Baginski et al.) (90)

Similar to the calcium determination, 60 L of water or aqueous standards (0.5, 1.0, 1.5, 1.75, 2.0, 2.5, 3.0 mM of Mg were first pipetted into empty borosilicate tubes.

(i) Magon: The Magon reagent (3 mL) of Baginski et al. (90) was then pipetted to the tubes containing either water or standards and vortexed for a few minutes to ensure thorough mixing. This was accompanied with the final addition of 3 mL of potassium hydroxide solution which was followed by thorough mixing and after standing at room temperature for 5 min, the absorbances were read at 548 nm.

(ii) Calmagite: In the case of Calmagite, 3 mL of potassium hydroxide buffer containing EGTA were pipetted to the tubes with thorough mixing before the final addition of 1 mL of Calmagite reagent. The mixtures were allowed to stand for 5 min and their absorbances read at 525 nm (76).

(iii) MTB: The addition of 3 mL of ammonium chloride/ammonium hydroxide buffer (78) with thorough mixing was

followed by the final addition of 3 mL of MTB reagent. The absorbances were determined against the reagent blank at 610 nm.

(c) Phosphate Determination (Method of Baginski et al. (100, 102))

To each 13 x 100 mm borosilicate test-tube, 50 µL of water or phosphate working standards (1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 mg/dL) were pipetted in triplicate. Then 0.5 mL of dilute sulfuric acid was added to each tube and vortexed. This was followed by the addition of 0.5 mL of A-SDS with thorough mixing before 0.5 mL of molybdate solution (AMA) was added.

After the tubes were vortexed for several seconds, they were left to stand for 1 or 5 min before 1.0 mL of arsenite-citrate solution (ACD) was added to the reaction mixture and vortexed again. After 10 min, the absorbances were then read against a reagent blank at 700 nm.

B. CLINICAL STUDIES

1. Equipment

These were similar to the ones described in ANALYTICAL STUDIES (CHAPTER II A.1, p. 37).

2. Materials

The same chemicals were used for the determinations of calcium, magnesium and phosphate in cerebrospinal fluid as

listed in ANALYTICAL STUDIES (CHAPTER II A. 2, p. 38).

3. Reagents

The reagents were made up as described in ANALYTICAL STUDIES (CHAPTER II A. 3, p. 40).

4. Methods

The same methods were used for the clinical studies as for the analytical studies except that pooled cerebrospinal fluid was substituted for the standards.

In addition, precision studies and recovery studies were performed on the CSF. The cerebrospinal fluid was collected and stored in the freezer (-4°C) for several days and was taken out and thawed when needed. Individual samples were pooled before being used in the actual determinations. The CSF samples were stable for several weeks if kept frozen. Within-day and day-to-day precision studies were carried out for each test and reagent. Calcium, magnesium and phosphate standards were added to the pooled samples for recovery studies.

CHAPTER III

RESULTS AND DISCUSSION

A. ANALYTICAL STUDIES

1. Calcium Determination

(a) CPC

Figure 3 shows the comparison of two calibration curves for CPC (with and without urea) in the determination of calcium. In both cases the buffer used was DEA. Linearity was observed up to 5.0 mM calcium (which was beyond the expected range of calcium in CSF) for CPC containing 5M urea.

With the addition of 3 mL of DEA buffer to the reaction mixture, a light violet colour was obtained for the blank while the colour changed from light to dark violet in direct proportion to the concentration of calcium.

CPC containing urea gave a higher absorbance with (ε = 2.018 x 10^6 mol cm^{-2}) calcium standards than CPC without urea (ε = 1.5117 x 10^6 mol cm^{-2}). Therefore, more detailed studies with the incorporation of urea were carried out. Thus, the only modification to the established method of Baginski et al. (53) was the addition of 5 M urea to the CPC reagent.

With this modification, sensitivity increased about 20% and correlation was 0.9976. However, in the preliminary

FIGURE 3

CALCIUM DETERMINATION USING CPC-COMPARISON OF TWO METHODS

Legend

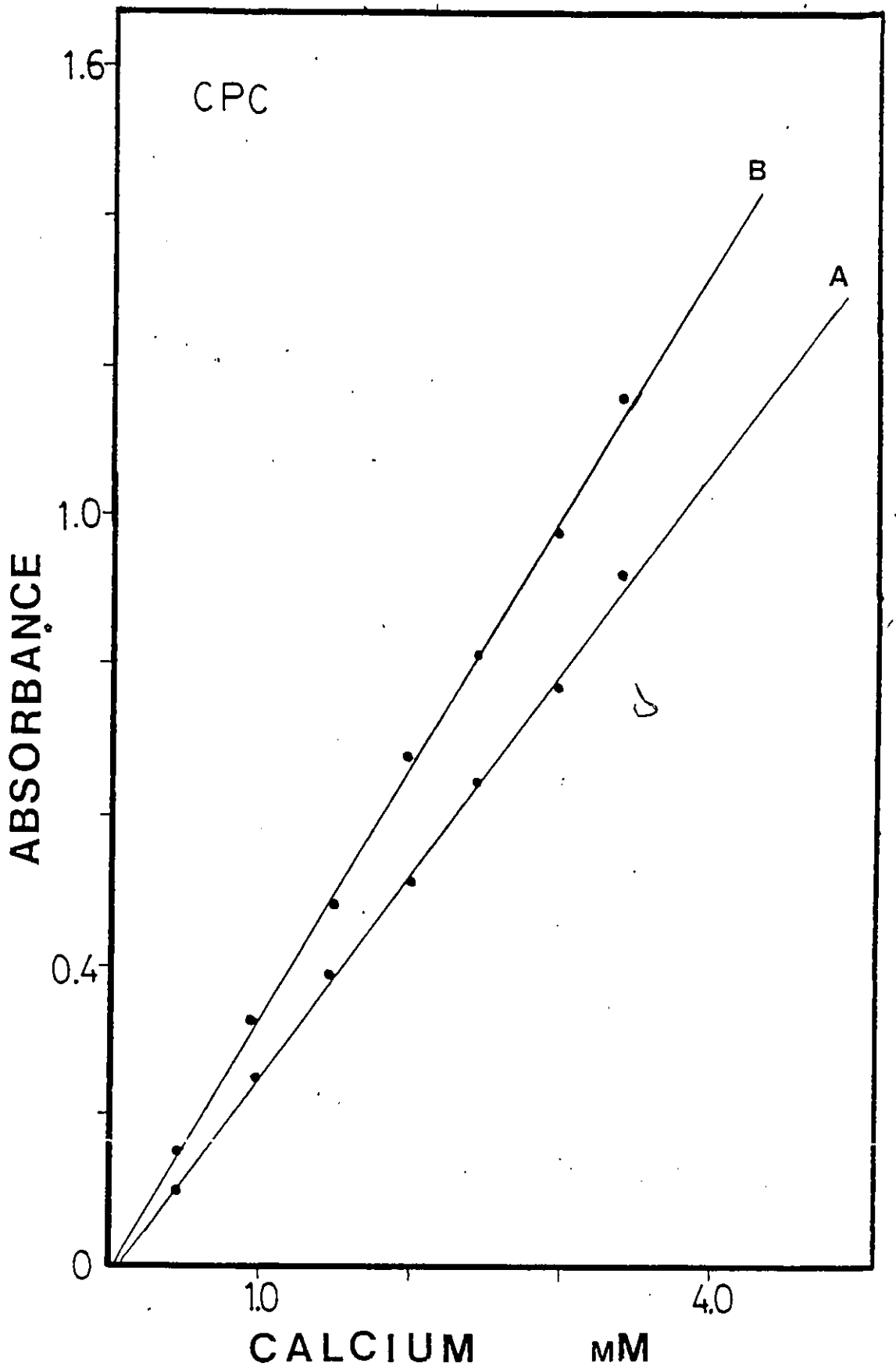
A. Standard curve of CPC (λ_{max} 575 nm) without urea is shown. The regression equation was $y = 0.2681x - 0.0165$ ($r = 0.9702$, $\varepsilon = 1.5117 \times 10^6$ mol cm^{-2}).

B. Standard curve of CPC (λ_{max} 575 nm) with urea is shown. The regression equation was $y = 0.3329x - 0.0133$ ($r = 0.99764$, $\varepsilon = 2.018 \times 10^6$ mol cm^{-2}). Linearity was observed till 4 mM Ca. The same DEA buffer was used for both determinations.

The molar absorptivity was reported by Sarkar and Chauhan (52) as 2.0×10^4 mol cm^{-2}. There was increased sensitivity with the modified technique of 100 fold.

Each point on the calibration curve is an average of triplicate experiments.

FIGURE 3



studies, when 5.0 M urea was dissolved in the buffer (63), there was no appreciable increase in absorbance when compared to DEA buffer alone used in conjunction with CPC containing urea. There was a decrease in the absorbance of the blank though this was counteracted by the lower absorbances of the standards. It seemed that the addition of urea to the CPC reagent alone was enough to cause an increase in absorbance of the standards. Thus, urea was omitted from the buffer.

Lorentz (63) used ethanol in addition to DMSO in their system in the preparation of the colour reagent. However, the original purpose was to keep the solvents similar and constant for comparison between the methods. This criterion would be overridden if extra solvent were introduced. Therefore, ethanol was eliminated from the system.

The results of the interference studies (Figure 4) showed that there was hardly any interference from magnesium when 8-hydroxyquinoline was incorporated in the reagent. In Figure 4A, the slopes of the lines coincided with each other showing high precision, while Figure 4B showed that at each identical calcium concentration the lines were horizontal. This showed that magnesium did not interfere at the physiological concentrations investigated. Even with high concentrations of magnesium, no positive interference could be detected. Thus CPC with

FIGURE 4

INTERFERENCE STUDIES USING CPC (WITH UREA)
IN THE DETERMINATION OF CALCIUM

Legend

Nine different combinations of Ca and Mg (given in Table VII) were determined in triplicate.

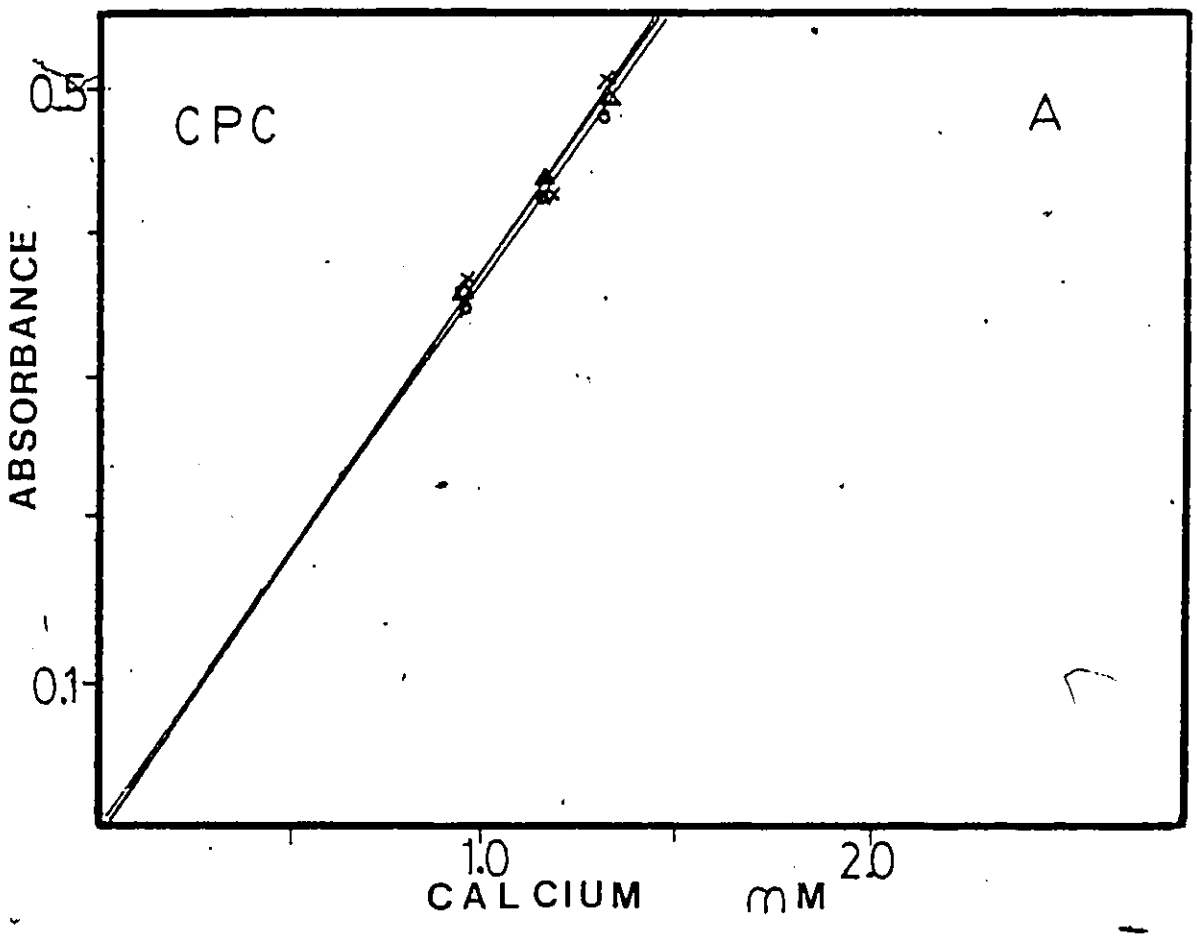
A: The absorbance was plotted against Ca concentration

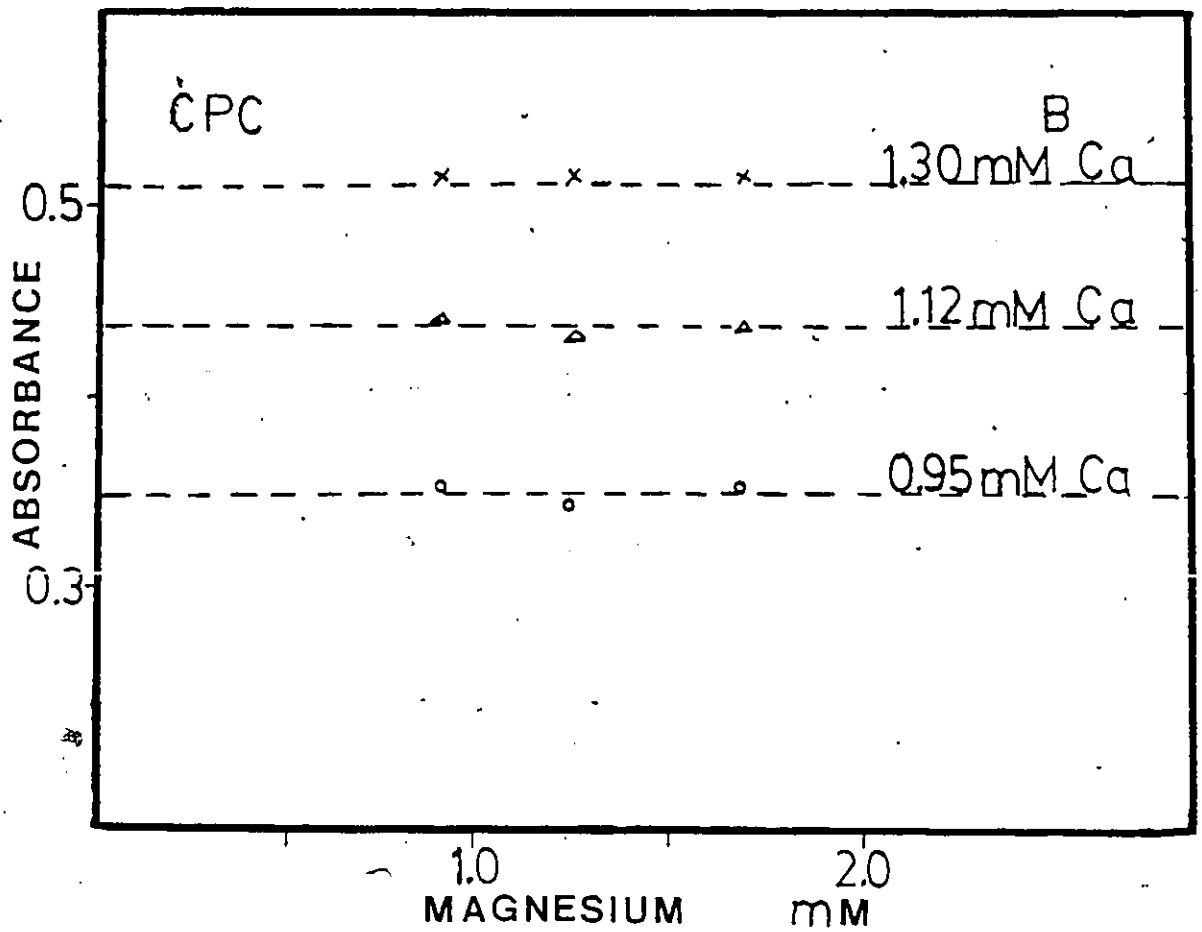
(o) represents A, B, C
(Δ) represents D, E, F
(×) represents G, H, I

B: The absorbance was plotted against Mg concentration

(o) represents A, D, G
(Δ) represents B, E, H
(×) represents C, F, I

FIGURE 4


CPC
A
0.5
0.1
ABSORBANCE
1.0
2.0
CALCIUM mM


CPC
B
1.30 mM Ca
1.12 mM Ca
0.95 mM Ca
0.5
0.3
ABSORBANCE
1.0
2.0
MAGNESIUM mM

urea (molar absorptivity, $\varepsilon = 2.018 \times 10^6$ mol cm^{-2}) was more sensitive than CPC alone ($\varepsilon = 1.5117 \times 10^6$ mol cm^{-2}) for the determination of calcium. The spectra of the standards against a water blank and reagent blanks are illustrated in Figure 5.

(b) Calmagite

The calibration curve of Calmagite in the determination of calcium is shown in Figure 6. Beer's law was obeyed till 3.0 mM calcium. As could be seen from the graph, Calmagite was less sensitive than CPC as the molar absorptivity was 3.5766×10^5 mol cm^{-2} compared to 2.018×10^6 mol cm^{-2} for CPC with urea.

Positive interference from magnesium is illustrated in Figure 7. Figure 7A showed that there was considerable interference from magnesium as the slopes of the lines were not parallel to each other and did not coincide. The reagent complexed with magnesium as well and thus was not completely masked with 8-hydroxyquinoline. Only 1.5 mL of Calmagite was used instead of 3 mL because the blank gave a very intense blue. As the concentration of Calmagite was almost 20 times as high as the original CPC concentration, it was concluded that enough colour reagent was present to chelate with the calcium.

Even when the concentration of 8-hydroxyquinoline was doubled, there was essentially some positive interference

FIGURE 5

SPECTRA OF CPC IN THE DETERMINATION OF CALCIUM

Legend

The spectra of CPC (containing urea) are shown λ_{max} 575 nm.

A: Reagent blank vs. water blank

B: 3.0 mM calcium vs. water blank

C: 3.0 mM calcium vs. reagent blank

D: CSF sample vs. reagent blank

The spectra of CPC were comparable to the spectra obtained by Parks, J. G. (1981), M.S. Thesis, University of Windsor. With the addition of 5 M urea, no change in the wavelength of maximum absorbance was observed.

FIGURE 5

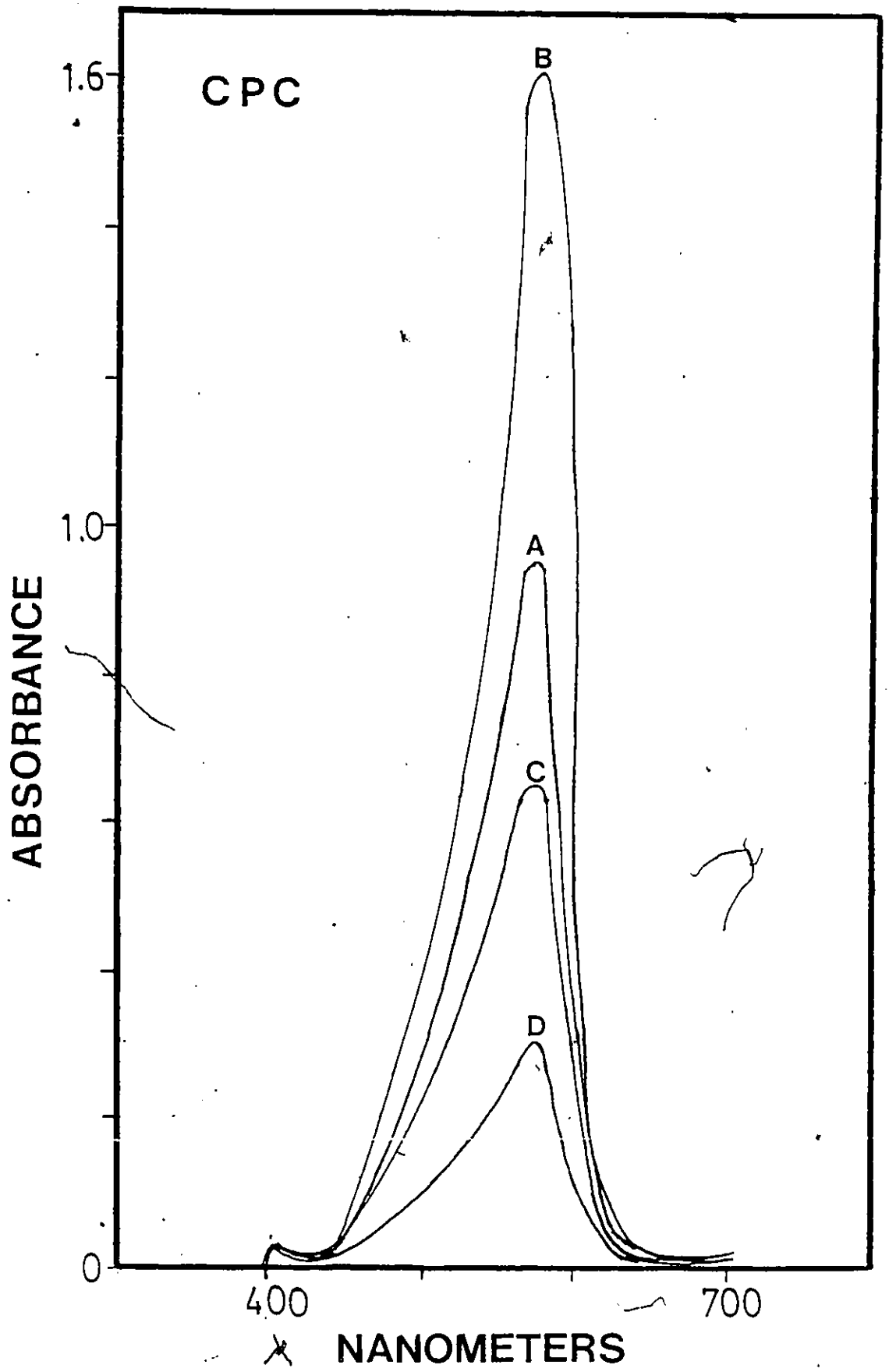
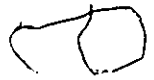


FIGURE 6

CALCIUM DETERMINATION USING CALMAGITE



Legend

Calibration curve of Calmagite is shown. λ_{max} 525 nm. The regression equations were:

A) $y = 0.969x + 0.0062$ ($r = 0.9933$, $\varepsilon = 3.5766 \times 10^5$ mol cm^{-2}), and B) $y = 0.0656x + 0.0024$ ($r = 0.9983$, $\varepsilon = 2.3383 \times 10^5$ mol cm^{-2}), respectively, when absorbances were taken 24 hours later. Linearity was observed up to 3.0 mM calcium.

Ingman and Ringbom (70) measured Calmagite-calcium complex at 520 nm but did not mention the molar absorptivity.

Each point on the calibration curve is an average of triplicate experiments.

FIGURE 6

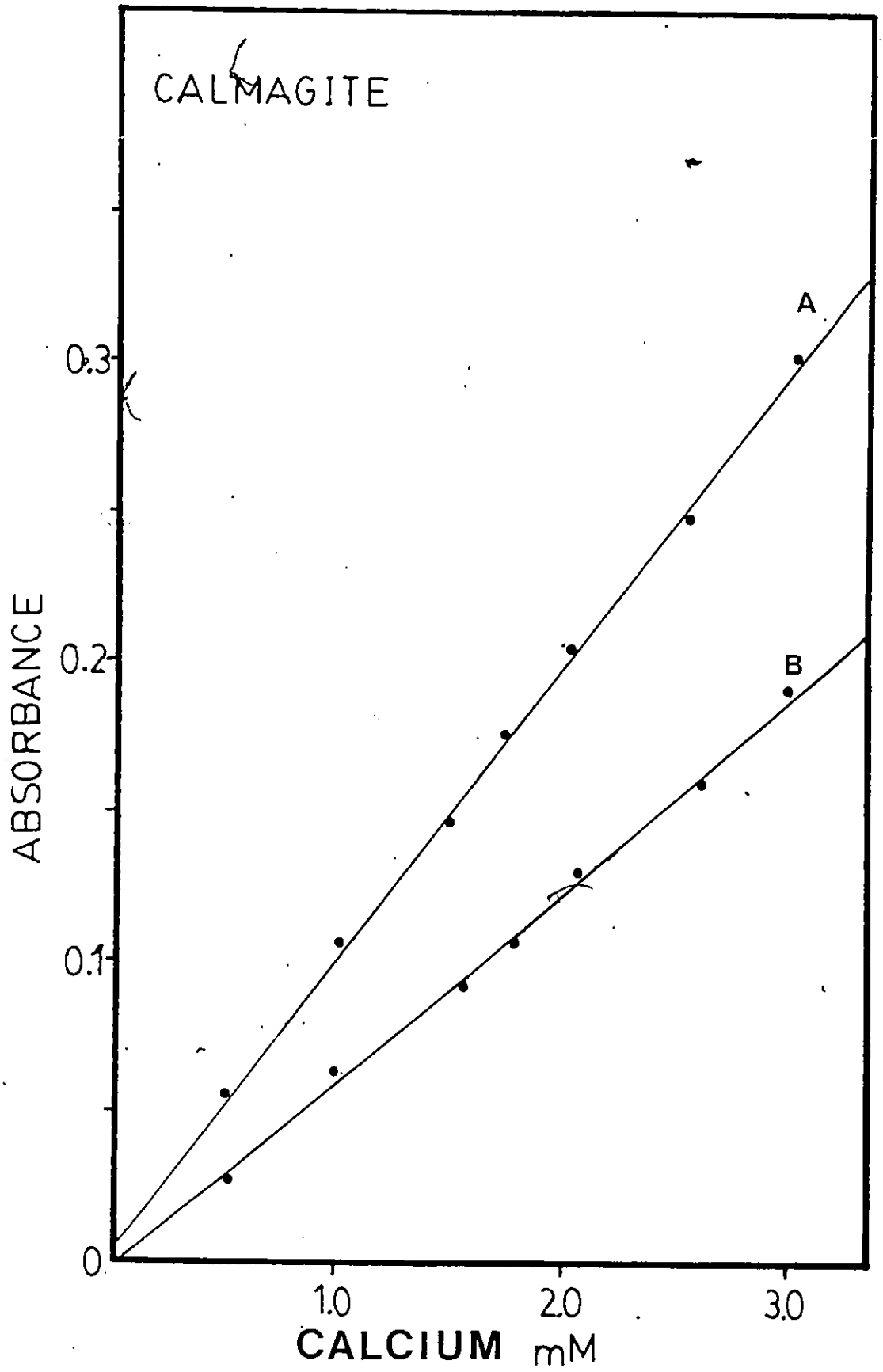


FIGURE 7

INTERFERENCE STUDIES USING CALMAGITE AND MTB IN THE DETERMINATION OF CALCIUM

Legend

Nine different combinations of Ca and Mg (given in Table VII) were determined in triplicate.

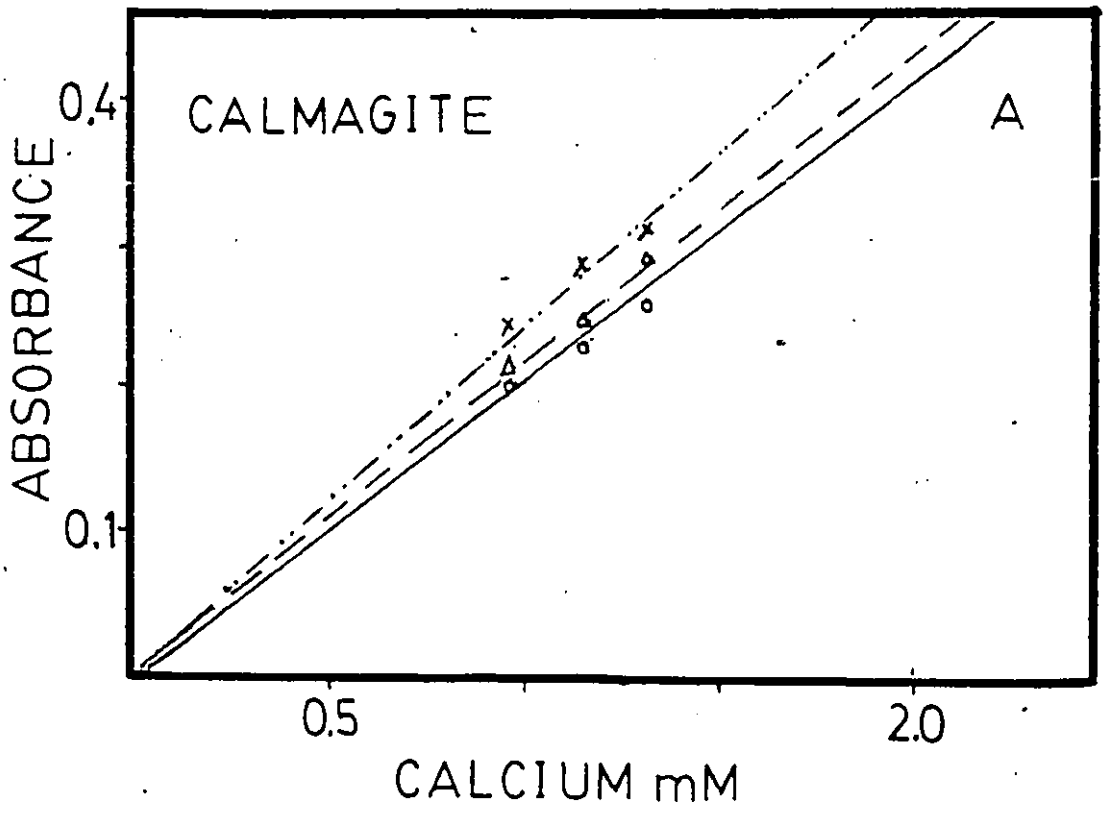
A: Positive interference from Mg could be seen using Calmagite as the reagent.

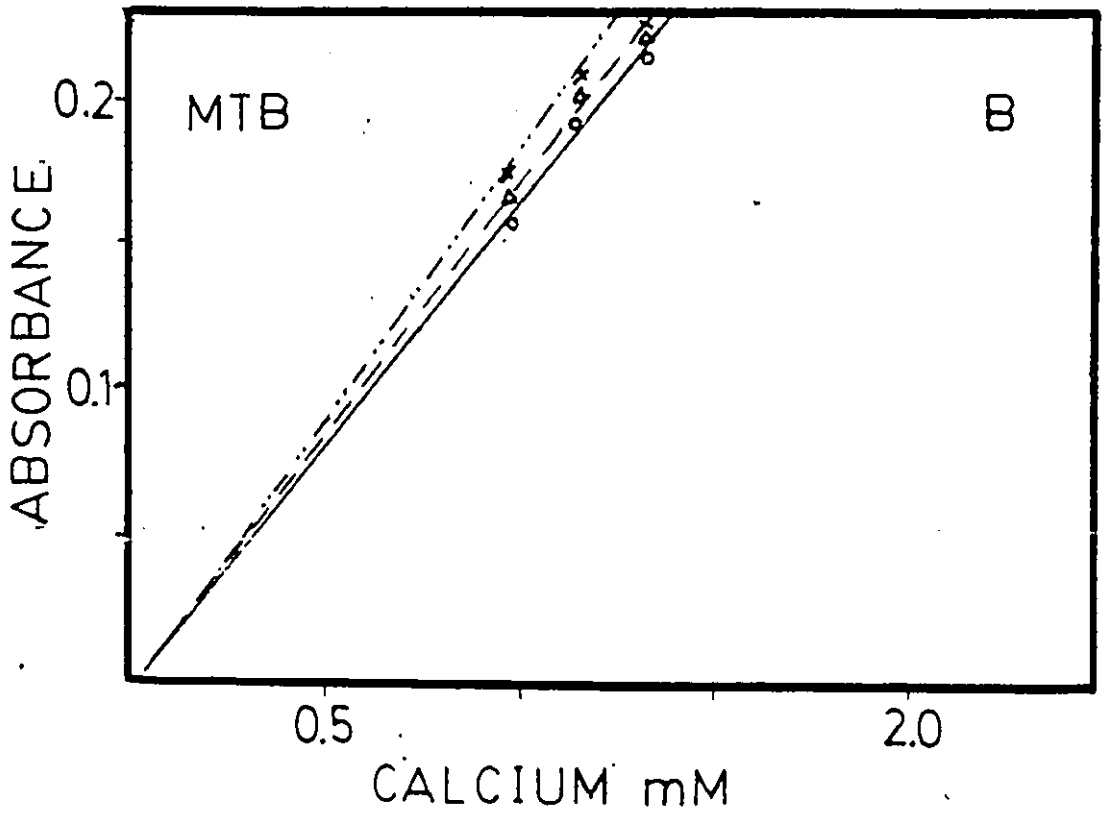
(o) represents A, B, C
(△) represents D, E, F
(×) represents G, H, I

B: Positive interference from Mg could be seen using MTB as the reagent.

(o) represents A, B, C
(△) represents D, E, F
(×) represents G, H, I

FIGURE 7


CALMAGITE
A
ABSORBANCE
0.4
0.1
0.5
2.0
CALCIUM mM


MTB
B
ABSORBANCE
0.2
0.1
0.5
2.0
CALCIUM mM

from magnesium. The results of the interference studies were compared with those of MTB (Figure 7B). Thus the additive effects of magnesium could not be entirely eliminated even if excess 8-hydroxyquinoline was present. Therefore, Calmagite was not very specific for calcium and could not be used for its determination as magnesium was a cause of considerable interference. Figure 8 is an illustration of the spectra of Calmagite against calcium.

(c) MTB

The calibration curve for MTB is given in Figure 9. Linearity was in the range of 0.0 - 3.0 mM calcium and the absorbance span was from 0.0 - 0.450. The concentration of MTB used was similar to the concentration of CPC, though the volume of DMSO was doubled because 8-hydroxyquinoline settled out of solution.

MTB was slightly more selective for calcium than Calmagite but its molar absorptivity (8.2528×10^5 mol cm^{-2}) was lower than CPC. Results of the interference studies can be seen in Figure 7. The interference from magnesium was not significant as the three lines lie practically on top of each other as illustrated in Figure 7B. The 8-hydroxyquinoline was enough to chelate nearly all the magnesium and there was hardly any positive interference. The reagent blank gave a yellow-green colour while the standard was greenish-blue.

FIGURE 8

SPECTRA OF CALMAGITE IN THE DETERMINATION OF CALCIUM

Legend

The spectra of Calmagite are shown. λ_{max} 510-525 nm λ_{max} of reagent blank was 600 nm.

A: Reagent blank vs. water blank

B: 3.0 mM calcium vs. water blank

C: 3.0 mM calcium vs. reagent blank

D: CSF sample vs. reagent blank

The λ_{max} of Calmagite-calcium complex was comparable to the λ_{max} of Calmagite-magnesium complex reported by Chauhan and Sarkar (76).

FIGURE 8

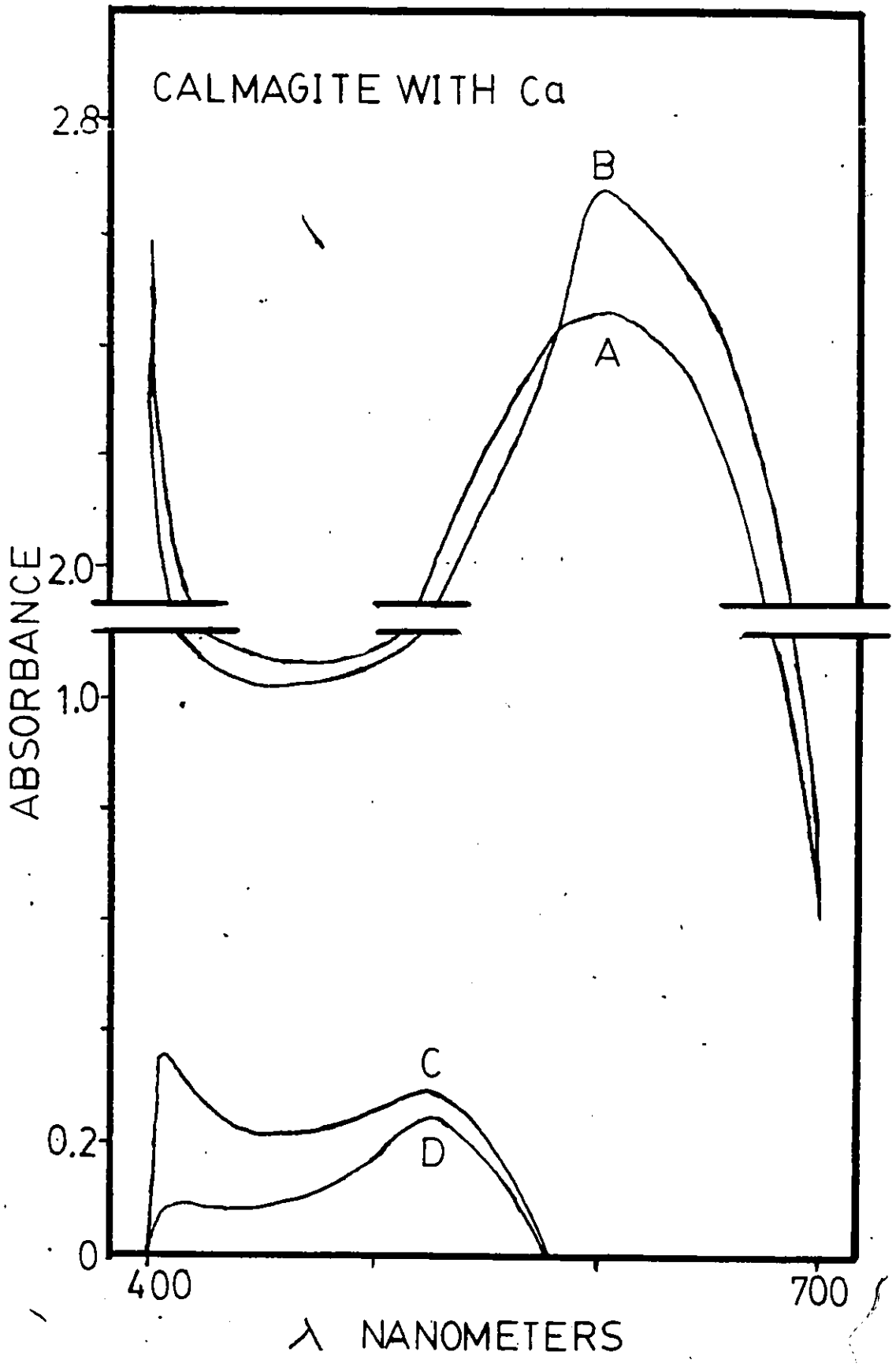

CALMAGITE WITH Ca
2.8
2.0
1.0
0.2
0
ABSORBANCE
B
A
C
D
400
700
λ NANOMETERS

FIGURE 9

CALCIUM DETERMINATION USING MTB

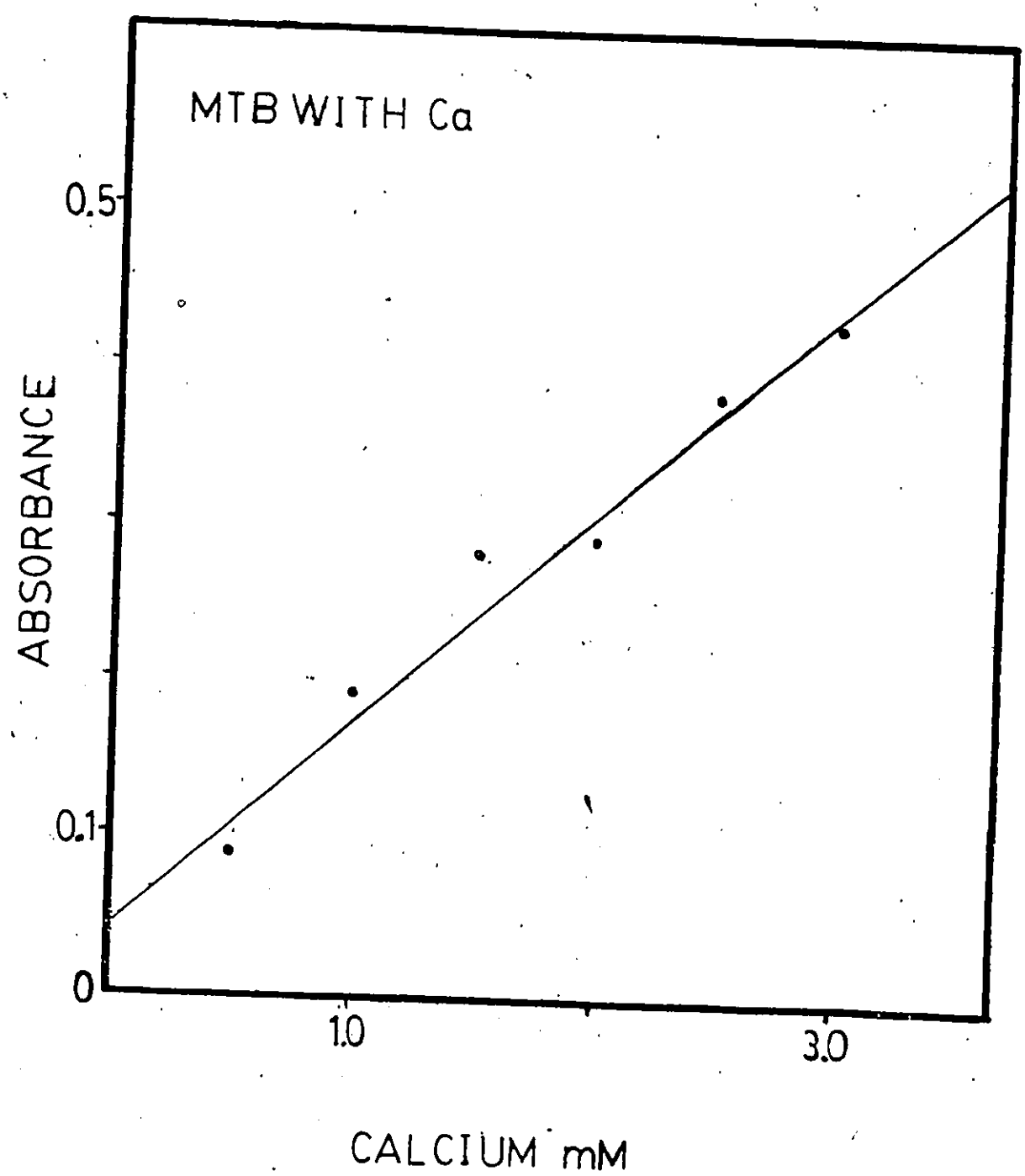
Legend

Calibration curve of MTB (λ_{max} 610 nm) is shown. The regression equation was $y = 0.1361x + 0.0428$ ($r = 0.9839$, $\varepsilon = 8.2528 \times 10^5$ mol cm^{-2}).

Metcalfe (78) reported a molar absorptivity of 1.520×10^4 mol cm^{-2} at 610 nm under ethanolic conditions, while MTB following the present conditions gave a higher molar absorptivity, 8.2528×10^5 mol cm^{-2}.

Each point on the calibration curve is an average of triplicate experiments.

FIGURE 9



Thus MTB was more specific for calcium than Calmagite (Figure 7) but it was not sensitive enough in comparison to CPC. Figure 10 illustrates the spectra of MTB against calcium.

2. Magnesium Determination

(a) Magon

The calibration curve of Magon is shown in Figure 11. The linear range extended to 3.5 mM magnesium which covered the physiological range of magnesium in CSF. The colour was relatively stable as after 24 h the absorbances were almost the same as the original. The high molar absorptivity (1.7652×10^6 mol cm^{-2}) indicated that Magon was a sensitive reagent.

Figure 12 illustrates the results of the interference studies. As can be seen from Figure 12A, all three lines were parallel and coincided with each other, indicating that calcium interference was not a problem. This fact is proven by Figure 12B which showed that at each different concentrations of calcium, identical magnesium levels still gave the same absorbance (shown by horizontal lines). There was hardly any interference from calcium showing that Magon was very specific for magnesium.

An interesting phenomenon occurred when the pH of the potassium hydroxide solution was adjusted to 12.0 by the addition of a few drops of concentrated hydrochloric acid

FIGURE 10

SPECTRA OF MTB IN THE DETERMINATION OF CALCIUM

Legend

The spectra of MTB (λ_{max} 610 nm) are shown

A: Reagent blank vs. water blank

B: 3.0 mM calcium vs. water blank

C: 3.0 mM calcium vs. reagent blank

D; CSF sample vs. reagent blank

The λ_{max} was comparable to that reported by Metcalfe (78) for MTB-magnesium complex.

FIGURE 10

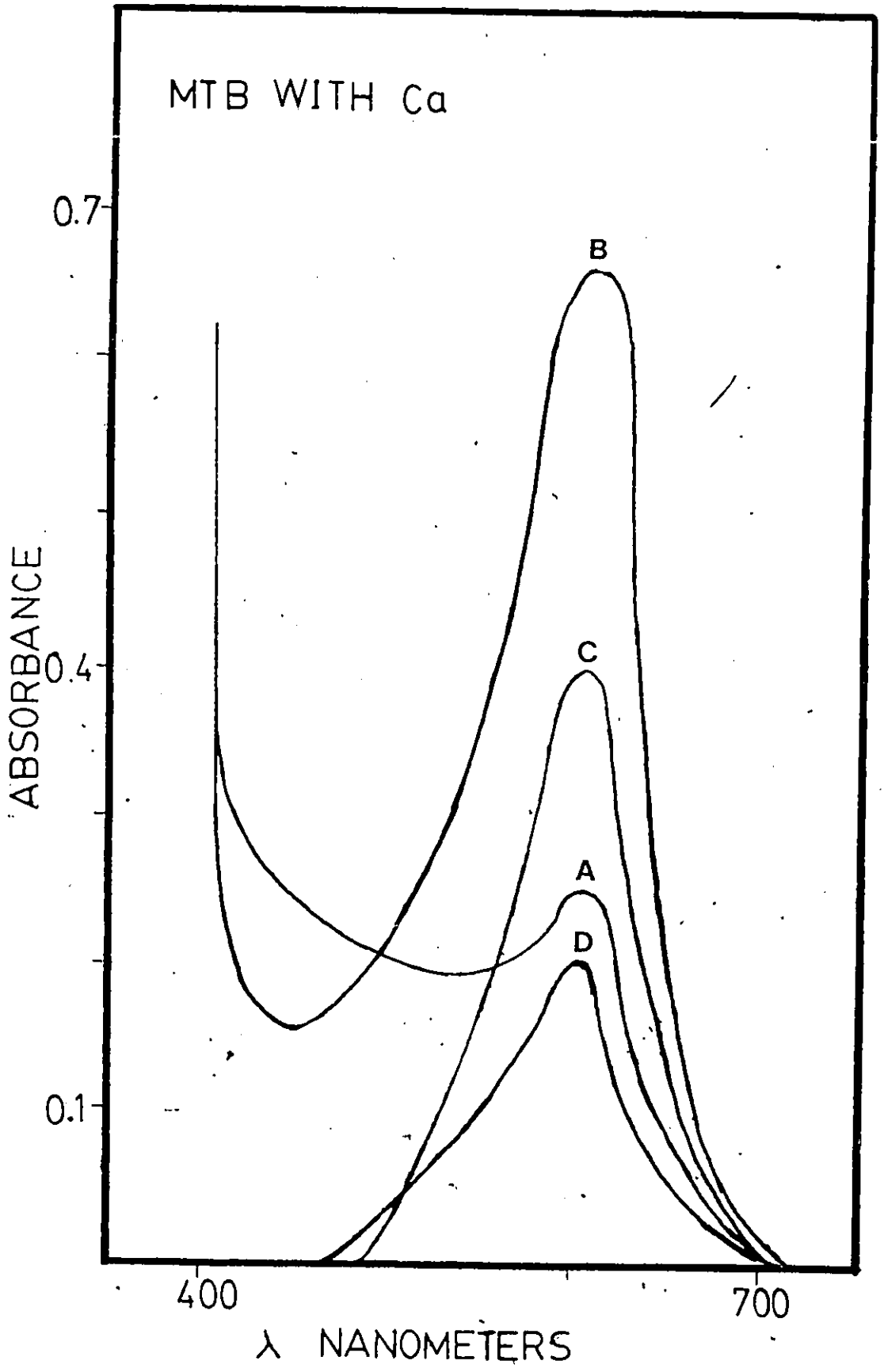


FIGURE 11

MAGNESIUM DETERMINATION USING MAGON

Legend

The calibration curve of Magon (548 nm) is shown. The regression equations were: A) $y = 0.2944x + 0.0105$ ($r = 0.9983$ $\varepsilon = 1.7652 \times 10^6$ mol cm^{-2}), and B) $y = 0.2858x + 0.0314$ ($r = 0.9946$, $\varepsilon = 1.7325 \times 10^6$ mol cm^{-2}) 24 hours later.

The molar absorptivity of Magon (1.7652×10^6 mol cm^{-2}) in the present study was higher than 3.04×10^4 mol cm^{-2} as reported by Chauhan and Sarkar (76).

Each point on the calibration curve is an average of triplicate experiments.

FIGURE 11

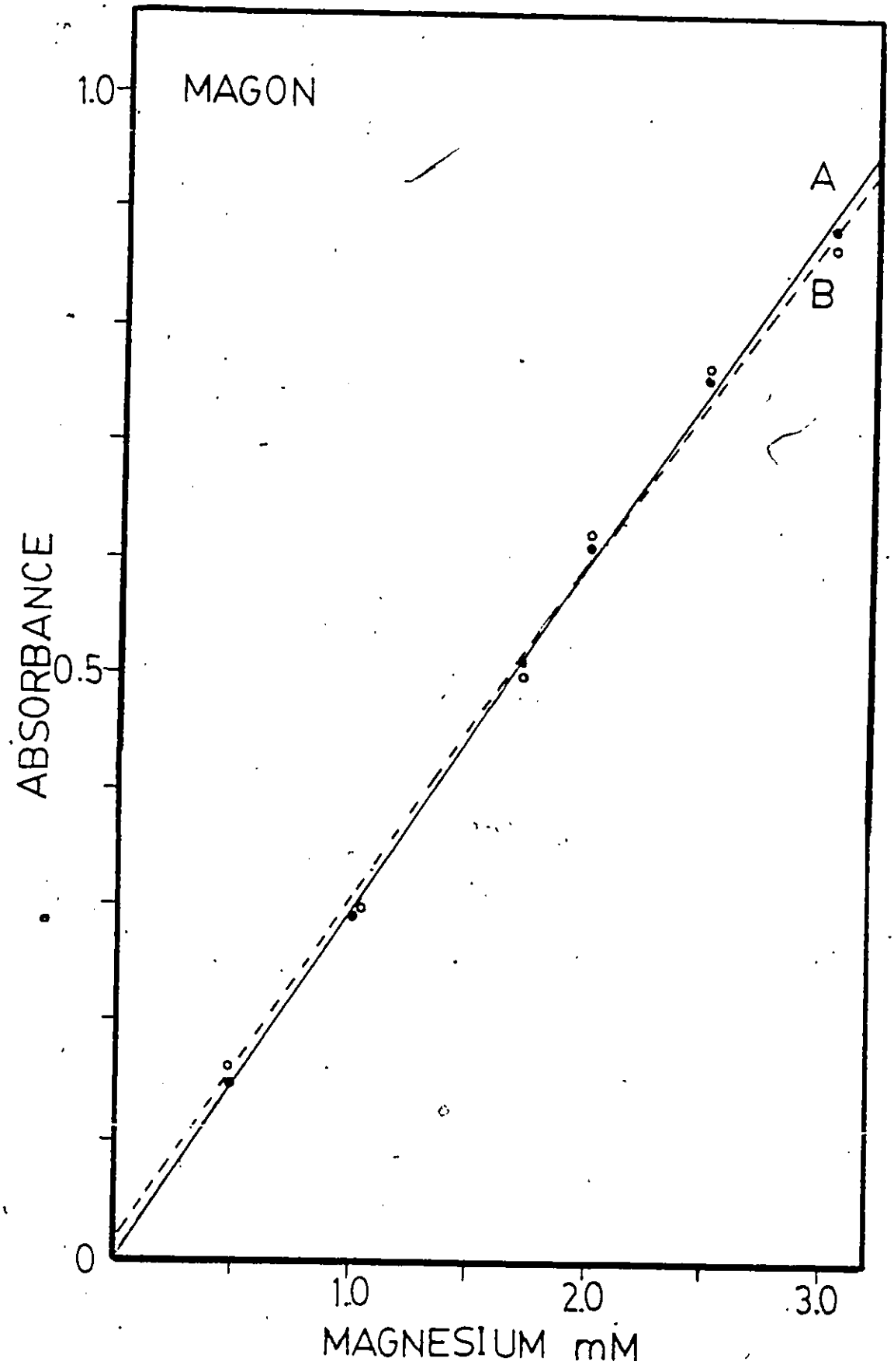


FIGURE 12

INTERFERENCE STUDIES WITH MAGON IN THE DETERMINATION OF MAGNESIUM

Legend

Nine different combinations of Ca and Mg (Table VII) were determined in triplicate.

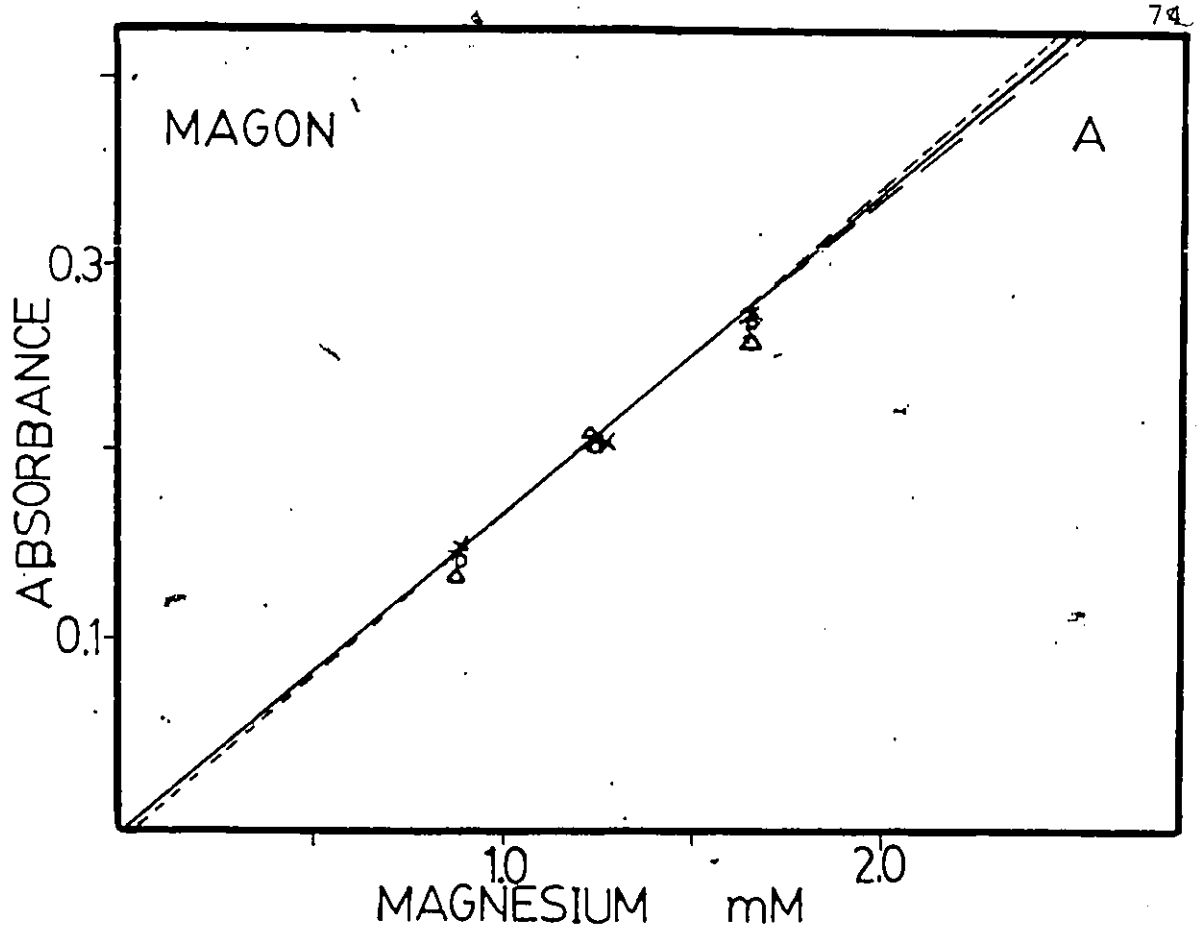
A: The absorbance was plotted against Mg concentrations.

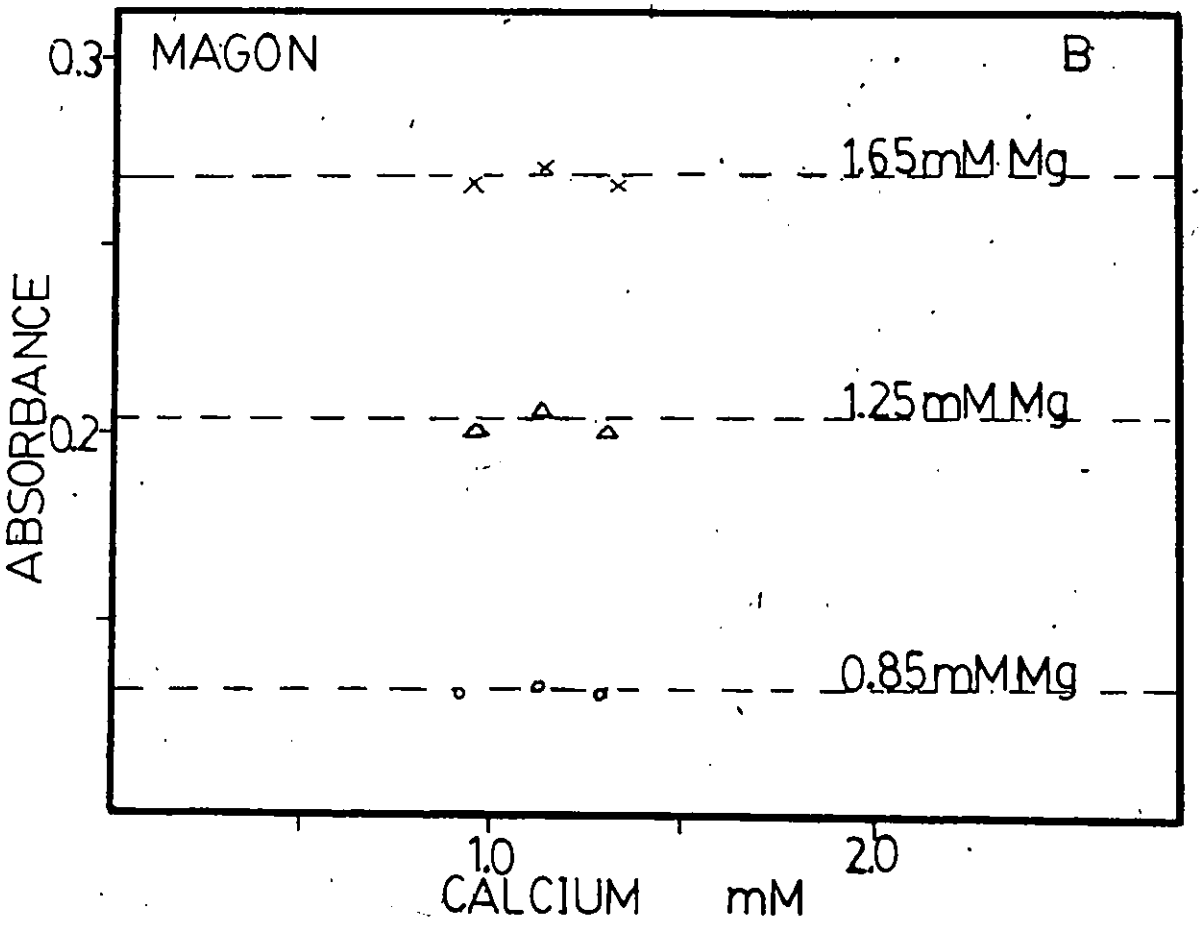
(o) represents A, D, G
(Δ) represents B, E, H
(×) represents C, F, I

B: The absorbance was plotted against Ca concentrations.

(o) represents A, B, C
(Δ) represents D, E, F
(×) represents G, H, I

FIGURE 12


MAGON
A
0.3
0.1
ABSORBANCE
1.0
2.0
MAGNESIUM mM


MAGON
B
0.3
0.2
ABSORBANCE
1.65mM Mg
1.25mM Mg
0.85mM Mg
1.0
2.0
CALCIUM mM

and the pH of the final mixture was 12.4. There was a hypochromic as well as a quenching effect in the absorbance maxima as illustrated in Figure 13. The peak at 548 nm disappeared and became the shoulder at 545 nm while there remained a maximum peak at approximately 518 nm. The normal spectra of Magon (with no adjustment of buffer pH) can be seen in Figure 14.

(b) Calmagite

Figures 15 and 16 show the calibration curve of Calmagite in the determination of magnesium and results of interference studies, respectively. Though potassium chloride-sodium hydroxide buffer (pH 12) was used by Chauhan and Sarkar (76), potassium hydroxide-potassium cyanide solution (90) was substituted to compare with the Magon procedure. The pH of the buffer system was very critical as only slight variations would affect the absorbances considerably. Thus the pH of the potassium hydroxide buffer was adjusted to 12.0 by adding a few drops of concentrated hydrochloric acid.

In this case, the potassium hydroxide solution containing EGTA was first added to the standards before the final addition of the colour. The use of the Magon base (KOH) proved to be superior to the original potassium chloride/sodium hydroxide buffer under the same conditions

FIGURE 13

COMPARISON OF MAGON SPECTRA AT DIFFERENT BUFFER pH'S

Legend

Two spectra are compared:

A: pH of KOH buffer was not adjusted to 12.0 by concentrated HCl (λ_{max} 518 and 548 nm). 3.0 mM magnesium vs. reagent blank.

B: pH of KOH buffer was adjusted to 12.0 by concentrated HCl (λ_{max} 518 nm). 3.0 mM magnesium vs. reagent blank.

Curve A is in agreement with the spectra reported by Baginski *et al.* (90).

FIGURE 13

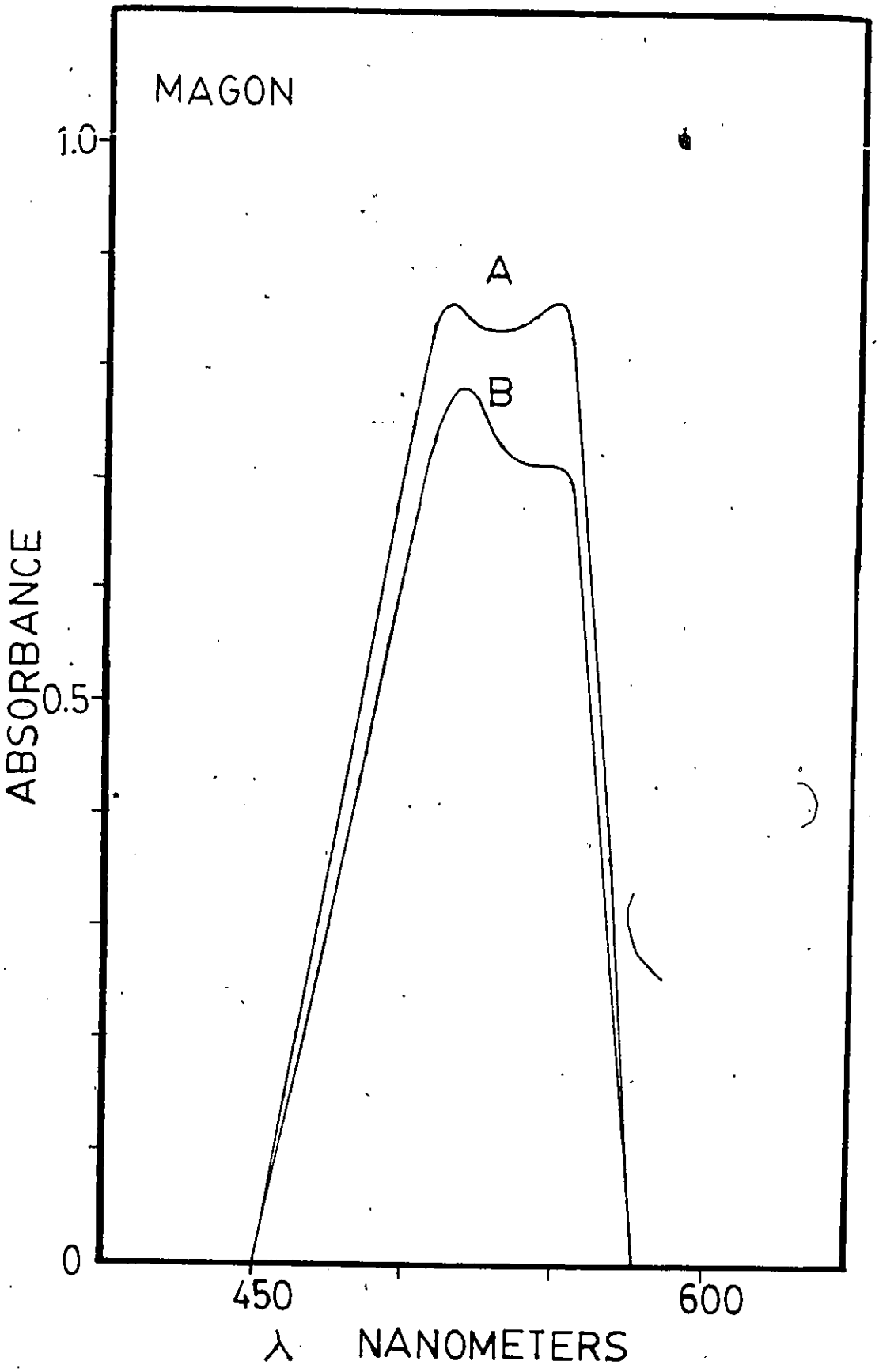


FIGURE 14

SPECTRA OF MAGON IN THE DETERMINATION OF MAGNESIUM

Legend

The spectra of Magon (λ_{max} 548 nm) is shown.

A: Reagent blank vs. water blank

B: 3.0 mM magnesium vs. water blank

C: 3.0 mM magnesium vs. reagent blank

D: CSF sample vs. reagent blank

The spectra are in agreement with the spectra reported by Baginski *et al*. (90).

FIGURE 14

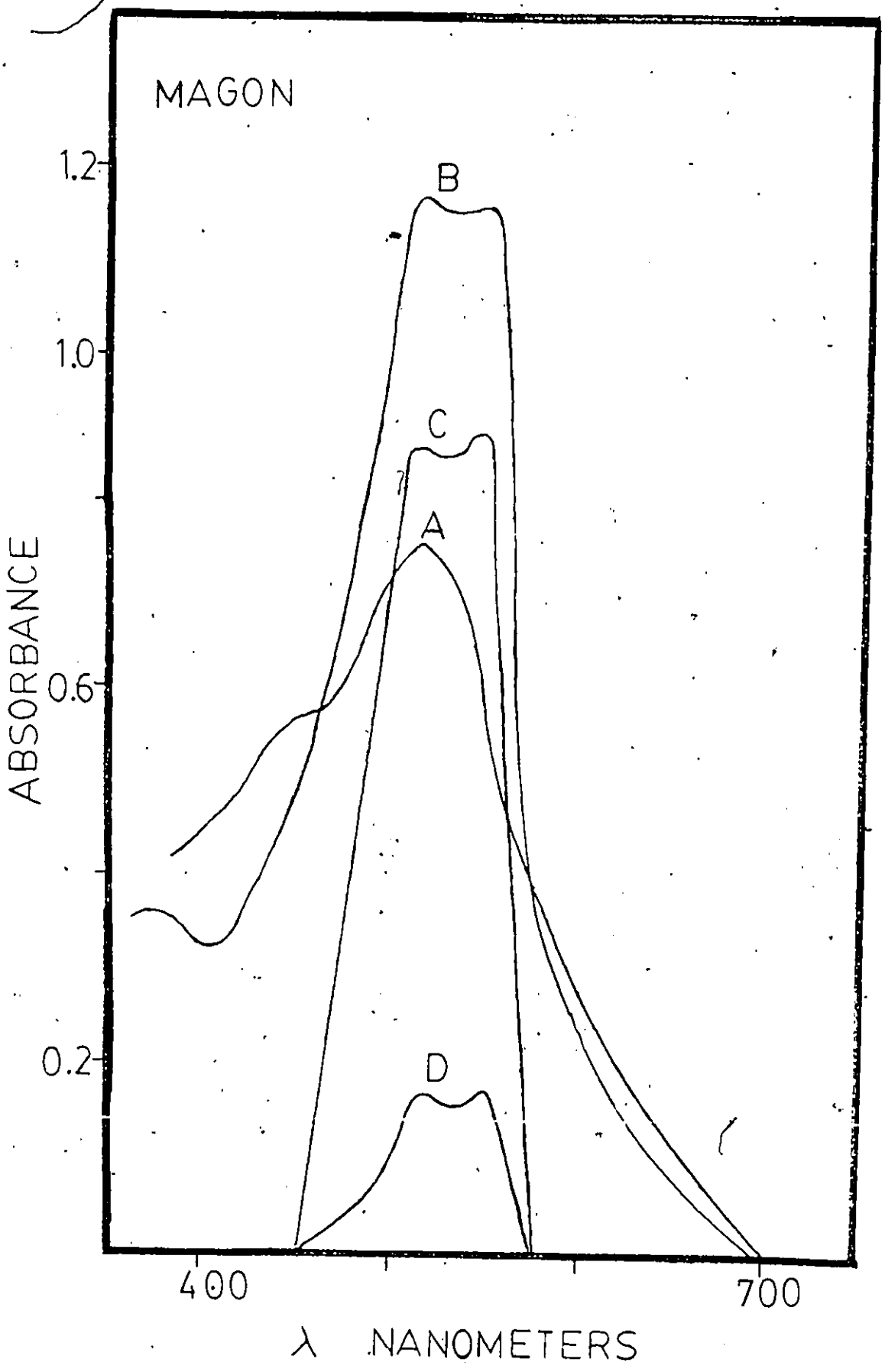


FIGURE 15

MAGNESIUM DETERMINATION USING CALMAGITE

Legend

Calibration curve of Calmagite (λ_{max} 525 nm) is shown. Buffer used was KOH/KCN (pH 12.0) containing EGTA.

The regression equations were: A) $y = 0.2478x - 0.0250$ ($r = 0.9927$, $\varepsilon = 1.0059 \times 10^6$ mol cm^{-2}), and B) $y = 0.2306x - 0.0236$ ($r = 0.9933$, $\varepsilon = 9.3665 \times 10^5$ mol cm^{-2}) 24 h later.

The molar absorptivity (1.0059×10^6 mol cm^{-2}) of Calmagite magnesium complex in the present study was higher than 1.74×10^4 mol cm^{-2} reported by Chauhan and Sarkar (76).

Each point on the calibration curve is an average of triplicate experiments.

FIGURE 15

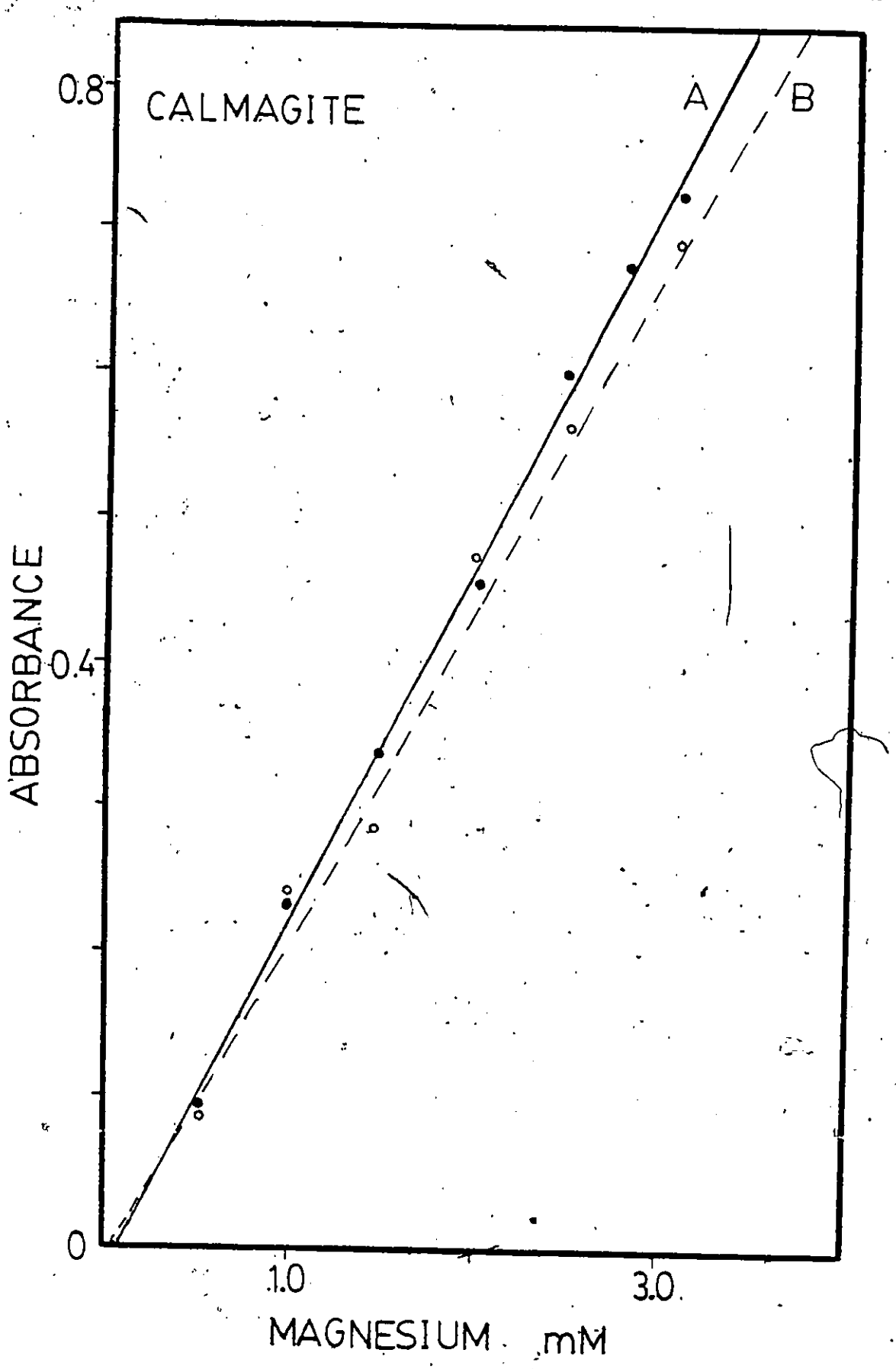


FIGURE 16

INTERFERENCE STUDIES USING CALMAGITE AND MTB IN THE DETERMINATION OF MAGNESIUM

Legend

Nine different combination of Mg and Ca (Table VII) were determined in triplicate.

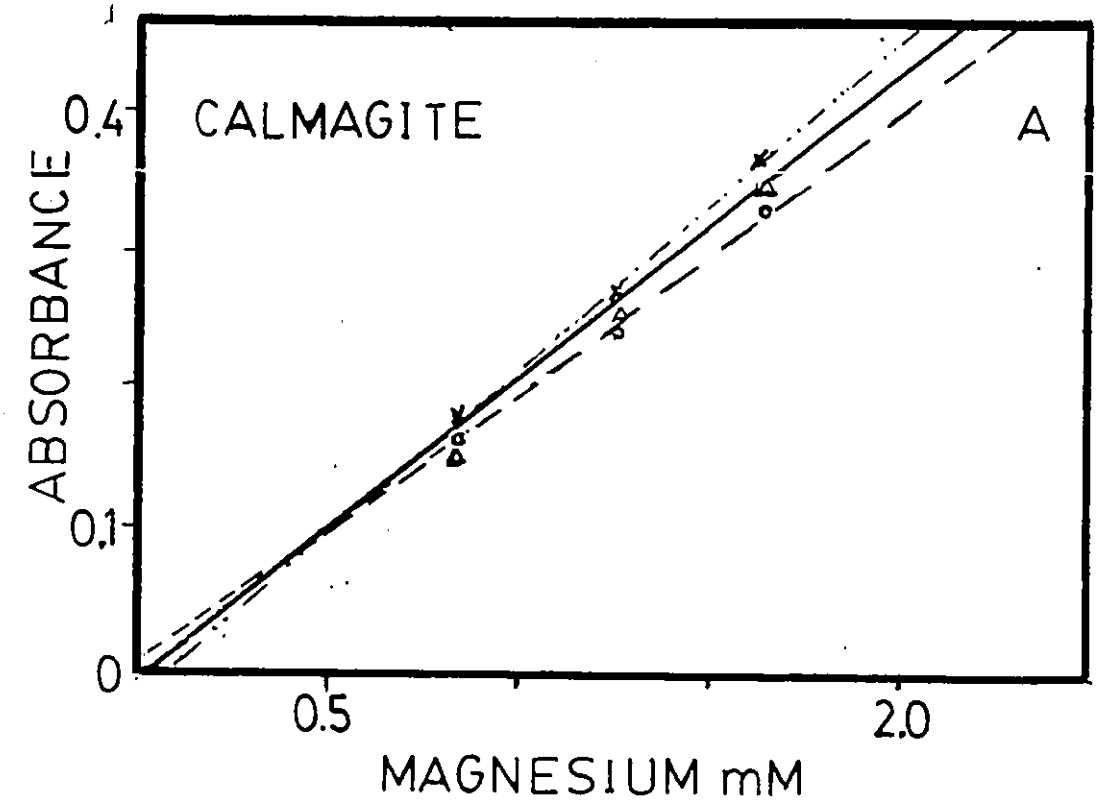
A: The absorbance was plotted against Mg concentration using Calmagite as the reagent. Some positive interference was noticeable.

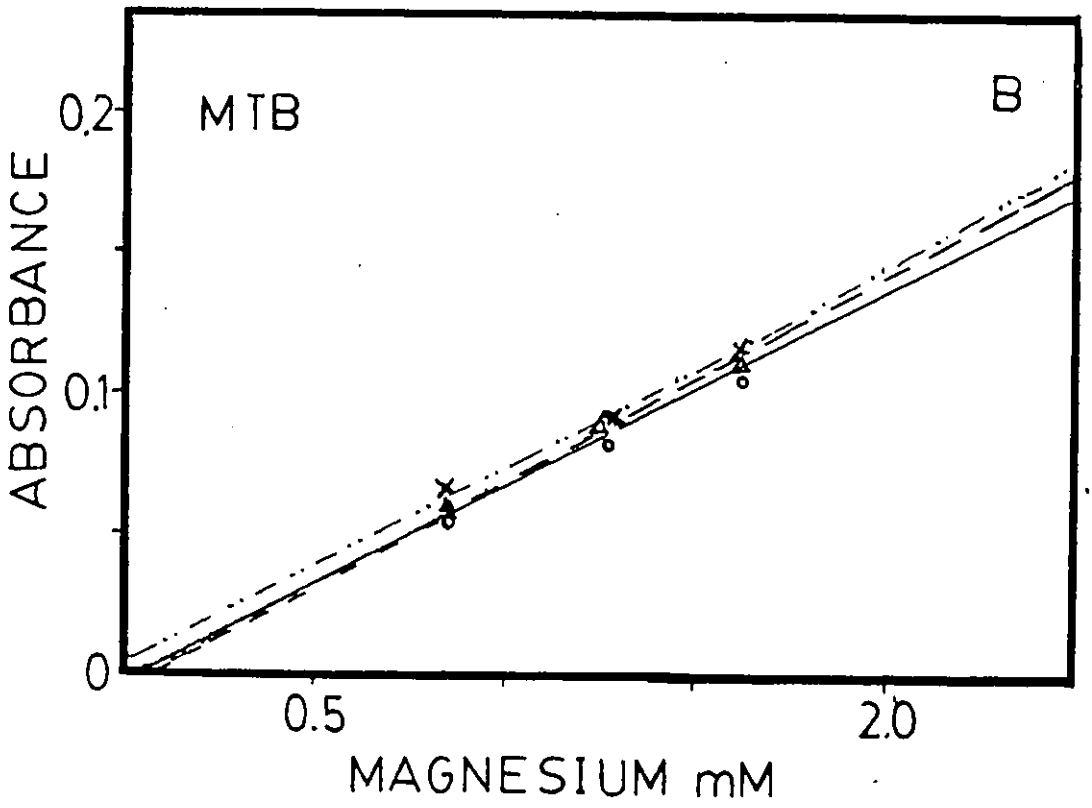
(o) represents A, D, G
(Δ) represents B, E, H
(x) represents C, F, I

B: The absorbance was plotted against Mg concentration using MTB as the reagent . Some interference from Ca could be seen as the lines were not exactly parallel or superimposed on each other.

(o) represents A, D, G
(Δ) represents B, E, H
(x) represents C, F, I

FIGURE 16


CALMAGITE
A
ABSORBANCE
0.4
0.1
0
0.5
2.0
MAGNESIUM mM


MTB
B
ABSORBANCE
0.2
0.1
0
0.5
2.0
MAGNESIUM mM

as the Magon procedure.

The reasons for this could be that DMSO was introduced into the system (90) whereas potassium chloride/ sodium hydroxide buffer worked best in an aqueous medium. Only 1 mL of Calmagite reagent was used in each determination because the concentration of Calmagite was almost twice the concentration of Magon used.

In an attempt to compare the 2 reagents by keeping their concentrations similar, Calmagite did not work very well at a concentration of 0.1947 mM. This meant that Calmagite only worked at a higher concentration than Magon, which proved that Magon was more sensitive for magnesium, even at a lower concentration. Optimum conditions were only obtained if the pH was kept at 12.0.

There was minimal calcium interference (Figure 16A) as the slopes of the lines were not exactly parallel. The results of the interference studies using MTB were almost similar to Calmagite (Figure 16B) indicating minimal calcium interference.

The spectra of Calmagite with magnesium are illustrated in Figure 17. It seemed to be a good reagent for the determination of magnesium as there was minimal interference from calcium which was chelated by EGTA. The main disadvantage was that the pH of the system was very critical for optimum conditions.

FIGURE 17

SPECTRA OF CALMAGITE IN THE DETERMINATION OF MAGNESIUM

Legend

The spectra of Calmagite (λ_{max} 525 nm) is shown. λ_{max} of reagent blanks was 600 nm.

A: Reagent blank vs. water blank

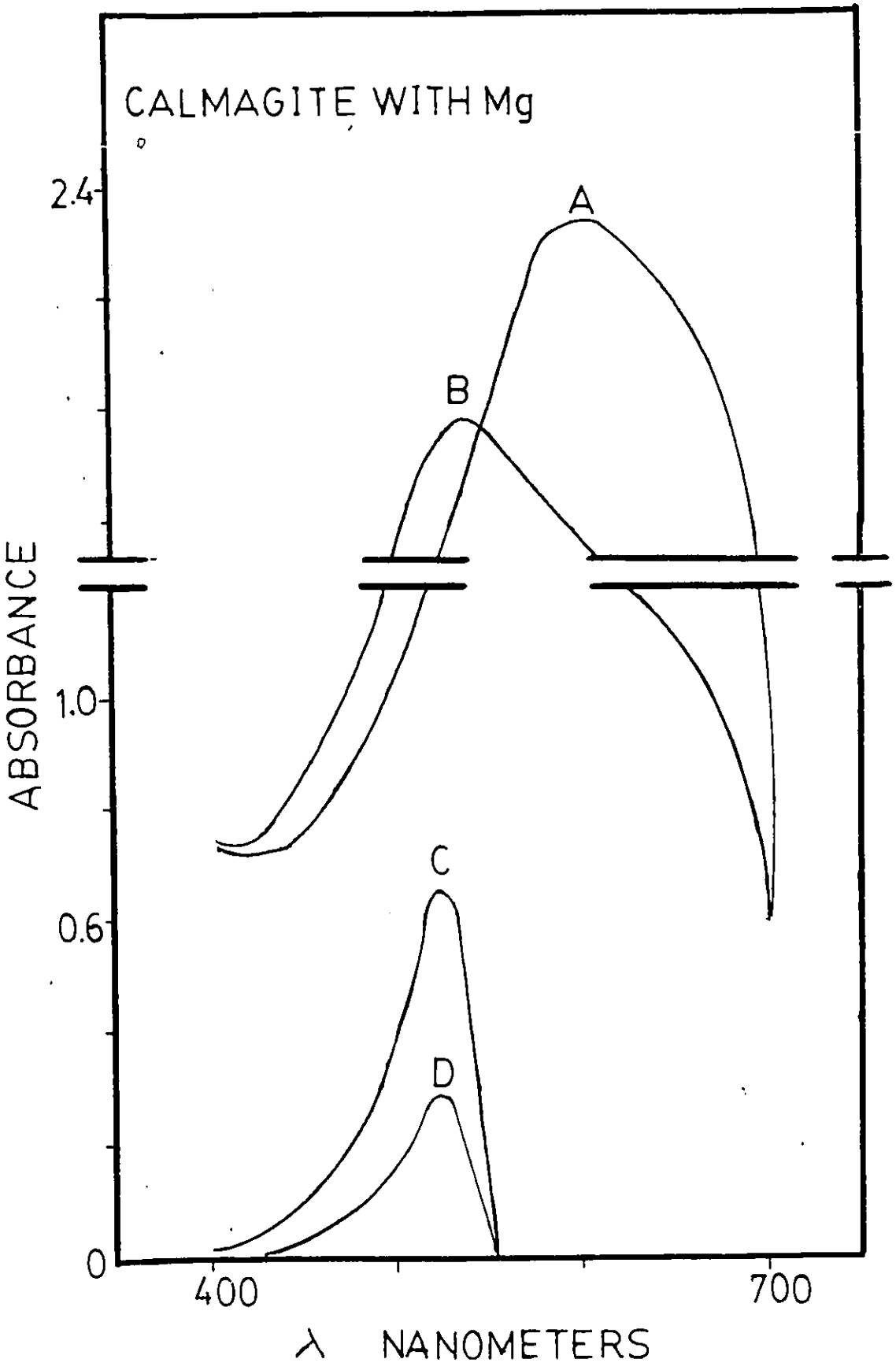
B: 3.0 mM magnesium vs. water blank

C: 3.0 mM magnesium vs. water blank

D: CSF sample vs. reagent blank

The absorption maximum of Calmagite-magnesium complex is in agreement with the results reported by Chauhan and Sarkar (76).

FIGURE 17


CALMAGITE WITH Mg
2.4
1.0
0.6
0
ABSORBANCE
A
B
C
D
400
700
λ NANOMETERS

(c) MTB

Figure 18 is an illustration of the calibration curve of MTB following the Magon procedure (90). The buffer used in this case was ammonium chloride/ammonium hydroxide (pH 10.8) as potassium hydroxide solution was not suitable for MTB under these conditions. There were hardly any absorption when potassium hydroxide was used. The blank gave a yellow-grey colour while the standards were a grey-blue colour. The pH was also very critical and thus had to be kept around 10.8.

Figure 16 gives the results of the interference studies for Calmagite and MTB. In Figure 16B, the slopes of the three lines were not parallel or identical to each other showing some interference from calcium. There was minimal interference from calcium as most of it would have been chelated with EGTA in the buffer. In this case, the buffer was added before the colour reagent as it contained EGTA.

The main disadvantages to this method were the low absorbance range 0.00 - 0.26 (for 0.0 - 3.5 mM magnesium) and the ammonia/ammonium chloride buffer system which were difficult to handle. Moreover, slight variations of pH resulted in large discrepancy of absorbances. Thus, precision was not good and accuracy was doubtful. The reagent was not sensitive for magnesium in comparison with

FIGURE 18

MAGNESIUM DETERMINATION USING MTB

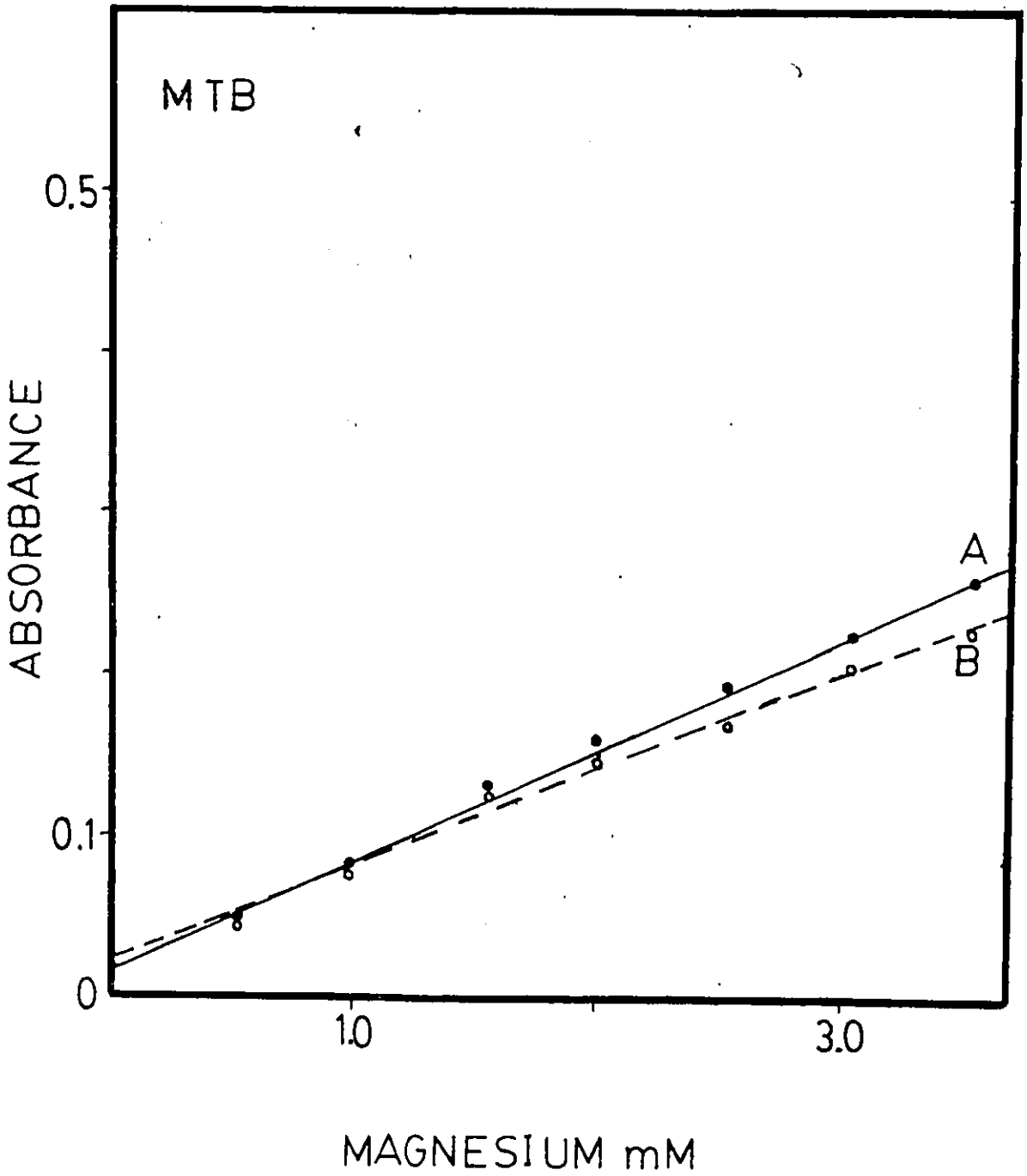
Legend

The calibration curve of MTB (λ_{max} 610 nm) is shown. Regression equations were: A) $y = 0.0724x + 0.0102$ ($r = 0.9974$, $\epsilon = 5.4302 \times 10^5$ mol cm^{-2}), and B) $y = 0.0605x + 0.021$ ($r = 0.9887$, $\epsilon = 4.5575 \times 10^5$ mol cm^{-2}), respectively, 24 h later. Buffer used was ammonia/ammonium chloride (pH 10.8) with EGTA.

The molar absorptivity (5.4302×10^5 mol cm^{-2}) is higher than 1.52×10^4 mol cm^{-2} as reported by Chauhan and Sarkar (76). Increase in sensitivity can be seen for MTB studied under present conditions. After 24 h there was a decrease in sensitivity of 15%.

Each point on the calibration curve is an average of triplicate experiments.

FIGURE 18



Magon. The spectra of MTB is illustrated in Figure 19.

3. Phosphate Determination

Figure 20 is the calibration curve of inorganic phosphate determination showing a good linear relationship, initially and even 24 hours later. The colour generated seemed to be stable for at least 24 hours, with only a 15% decrease in sensitivity. Beer's law was obeyed up to nearly 6.0 mg/dL of phosphate and there were no major interferences from the other constituents normally present in serum or CSF. The phosphomolybdate technique was a very specific and sensitive technique for inorganic phosphorus.

The colour of the blank was a light yellow while that of the standards ranged from light to dark blue depending on the concentration of phosphate. Figure 21 shows the spectra for phosphate determination.

B. CLINICAL STUDIES

1. Calcium Determination

The results of the recovery studies are given in Table VIII. It can be seen that recovery was excellent for CPC (97 to 102%), but was not proportional for Calmagite or MTB. There was a wide margin for error in the latter cases as the concentrations may be beyond the range of linearity.

FIGURE 19

SPECTRA OF MTB IN THE DETERMINATION OF MAGNESIUM

Legend

The spectra of MTB (λ_{max} 610 nm) are shown.

A: Reagent blank vs. water blank

B: 3.0 mM magnesium vs. water blank

C: 3.0 mM magnesium vs. reagent blank

D. CSF sample vs. reagent blank

The absorption maxima is in agreement with the value reported by Metcalfe (78).

FIGURE 19

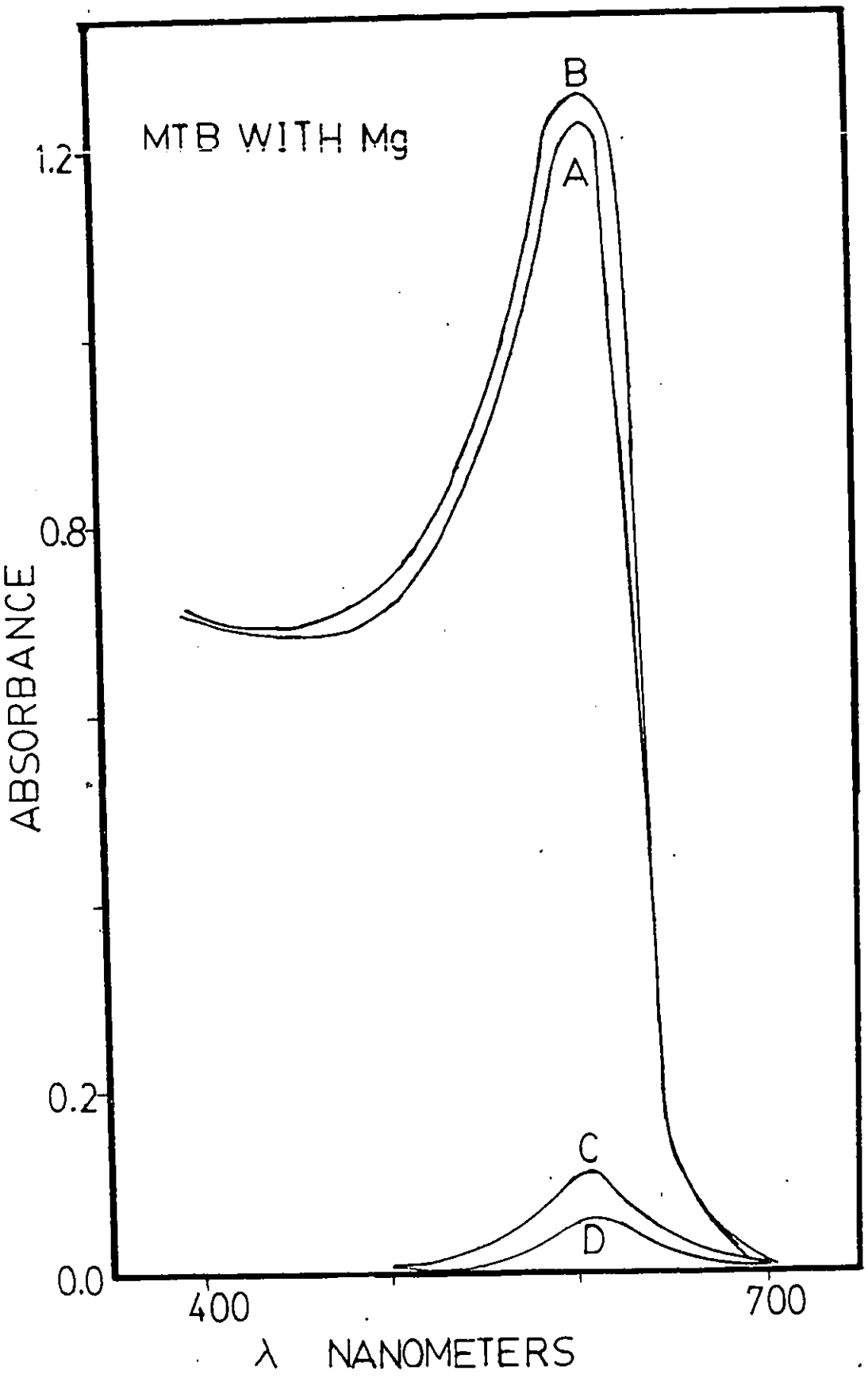


FIGURE 20

INORGANIC PHOSPHATE DETERMINATION

Legend

The calibration curve of inorganic phosphate is shown (λ_{max} 700 nm). Linearity was obeyed till 6.0 mg/dL of phosphate.

The regression equations were: A) $y = 0.1112x - 0.0136$ ($r = 0.9986$, $\varepsilon = 8.7851 \times 10^5$ mol cm^{-2}), and B) $y = 0.101x - 0.0072$ ($r = 0.9980$, $\varepsilon = 7.9835 \times 10^5$ mol cm^{-2}), respectively, 24 h later.

There was about a 9% decrease in molar absorptivity after 24 h.

Each point on the calibration curve is an average of triplicate experiments.

FIGURE 20

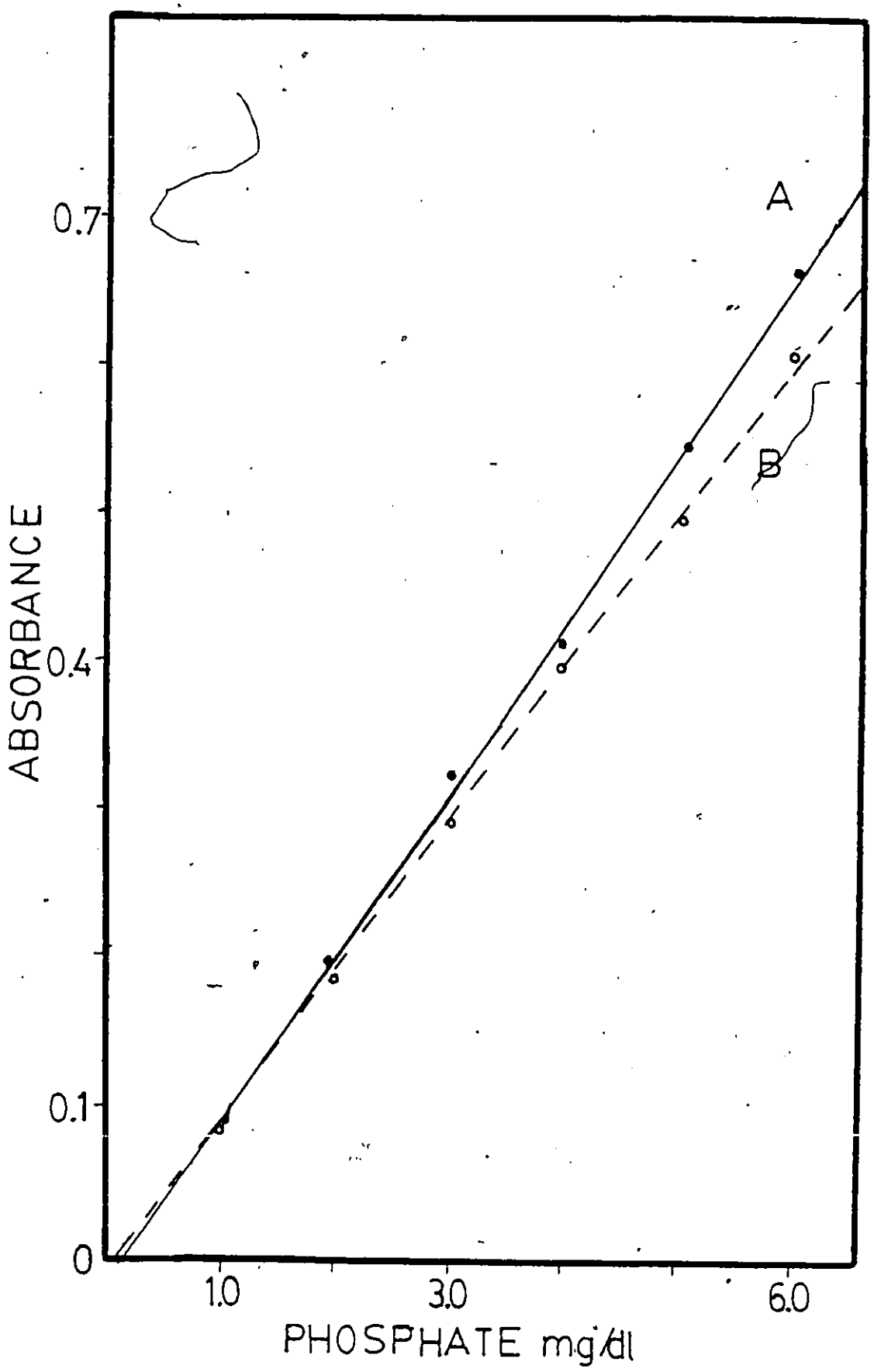


FIGURE 21

SPECTRA OF INORGANIC PHOSPHATE DETERMINATION

Legend

The spectra of phosphomolybdate complex are shown (λ_{max} 700 nm).

A: Reagent blank vs. water blank

B: 3.0 mg dL^{-1} phosphate vs. water blank

C: 3.0 mg dL^{-1} phosphate vs. reagent blank

D: CSF sample vs. reagent blank

E: 6.0 mg dL^{-1} phosphate vs. reagent blank

The spectra are in agreement with those reported by Baginski *et al.* (102).

FIGURE 21

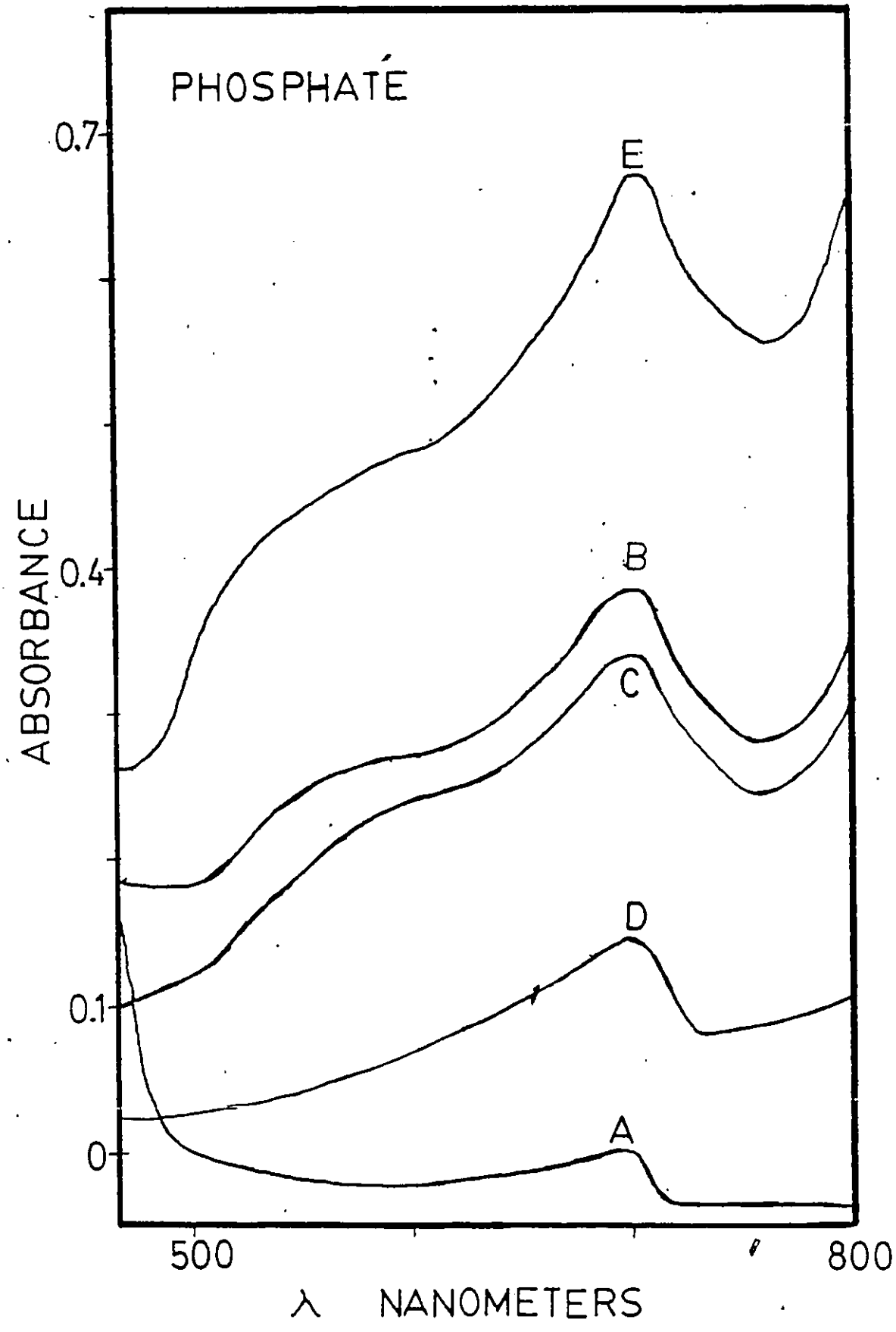


TABLE VIII

RECOVERY STUDIES FOR CALCIUM WITH CRESOLPHTHALEIN COMPLEXONE, CALMAGITE AND METHYLTHYMOL BLUE[a]

Reagents	Calcium present mM[b]	Calcium added mM[b]	Calcium found	% Recovery
CPC	0.98	-	-	-
		1.00	1.93	97
		2.00	3.02	102
		5.00[c]	-	-
Calmagite	1.00	-	-	-
		1.00	2.05	105
		2.00	3.30	115
		5.00[c]	-	-
MTB	0.95	1.00	1.72	81
		2.00	2.37	71
		5.00[c]	-	-

[a]These studies were made with pooled CSF. Calcium standards were added to the samples and analyzed.

[b]All results were the average of triplicate analyses.

[c]The absorbances were too high at that concentration which extends beyond the range of linearity.

Precision studies were carried out for within-run and day-to-day analyses (Table IX). The average coefficient of variation (within-run) for CPC, Calmagite and MTB were 1.07, 5.43 and 4.50%, respectively. CPC had very good precision in comparison with the others because it had the lowest C.V. while the standard deviation (S.D.) ranged from ±0.008 to ±0.027. The day-to-day analyses confirmed that CPC was the best reagent for calcium determination because the C.V. and S.D. gave the lowest value compared to the other two reagents.

2. Magnesium Determination

Table X shows the results of recovery studies for magnesium with the three reagents. Magon and Calmagite showed good recovery for Mg, but MTB was not very accurate as the percentage recovered ranged from 95-120%.

The results of the precision studies for magnesium can be seen in Table XI. For within-run analyses, Magon gave an average C.V. of 1.09% while Calmagite and MTB gave values of 2.62 and 5.54%, respectively. In day-to-day analyses, Magon was better than the other two reagents where precision was concerned with a C. V. of 1.15%.

3. Phosphate Determination

The results of the recovery and precision studies are given in Table XII. It was found that the phospho-

TABLE IX

PRECISION STUDIES FOR CALCIUM WITH CRESOLPHTHALEIN COMPLEXONE, CALMAGITE AND METHYLTHYMOL BLUE IN DAY-TO-DAY ANALYSES[a]

Reagents	n[b]	$\bar{X}$[c]	S.D.	C.V.	S.E.M.
CPC	3	0.853	±0.012	1.40	0.006
Calmagite	3	0.862	±0.043	4.98	0.024
MTB	3	0.988	±0.085	8.60	0.049

[a]The pooled samples were analyzed over a period of 3 days. Pooled CSF were kept at 4°C for several days.

[b]n was the number of runs. Each run consisted of an average of 10 analyses.

[c]The mean was an average of the means of each individual run.

TABLE X

RECOVERY STUDIES FOR MAGNESIUM WITH MAGON, CALMAGITE AND METHYLTHYMOL BLUE[a]

Reagents	Magnesium present mM[b]	Magnesium added mM[b]	Magnesium found	% Recovery
Magon	1.10	-	-	-
		1.00	2.10	100
		2.00	3.05	98
		5.00[c]	-	-
Calmagite	1.05	-	-	-
		1.00	1.90	90
		2.00	3.15	105
		5.00[c]	-	-
MTB	1.20	-	-	-
		1.00	2.40	120
		2.00	3.10	95
		5.00[c]	-	-

[a]These studies were made with pooled CSF. Magnesium standards were added to the samples and analyzed.

[b]All results were the average of triplicate analyses

[c]With the addition of 5 mM Mg to CSF, the absorbances were too high and beyond the range of linearity.

TABLE XI

PRECISION STUDIES FOR MAGNESIUM WITH MAGON, CALMAGITE AND METHYLTHYMOL BLUE IN DAY-TO-DAY ANALYSES[a]

Reagents	n[b]	$\bar{X}$[c]	S.D.	C.V.	S.E.M.
Magon	3	1.30 ±	0.015	1.15	0.008
Calmagite	3	1.30 ±	0.050	3.84	0.028
MTB	3	1.10 ±	0.095	8.63	0.054

[a]The pooled samples were analyzed over a period of 3 days. Pooled CSF were kept at -4°C for several days.

[b]n was the number of runs. Each run consisted of an average of 10 analyses.

[c]The mean was an average of the means of each individual run.

TABLE XII

RECOVERY[a,b] AND PRECISION STUDIES FOR PHOSPHATE DETERMINATION

Recovery studies

Phosphate present mg dL^{-1}	Phosphate added mg dL^{-1}	Phosphate found mg dL^{-1}	% Recovery
0.90	-	-	
	2.00	2.89	100
	5.00	6.25	107

Day-to-day precision

n[c]	$\bar{X}$	S.D.	C.V.	S.E.M.
3	1.32	± 0.026	1.96	0.015

[a]These studies were made with pooled CSF. Phosphate standards were added to the samples and analyses.

[b]All results were the average of triplicate analyses.

[c]The pooled samples were analyzed over a period of 3 days. Pooled CSF were kept at -4°C for several days. n was the number of runs and each run consisted of 10 analyses.

molybdate technique was very accurate for phosphate determination as the results agreed well with expected values. The recovery ranged from 100 to 107% and linearity was observed even at high concentrations.

These results confirmed that the method was very accurate and sensitive for inorganic phosphate determination. The average C.V. for within-run analyses was 1.35% and the S.D. ranged from ±0.010 to ±0.022. However, for the day-to-day analyses, the C.V. was 1.96% with a S.D. of ±0.026. Thus, one could conclude that the phosphomolybdate technique was highly suitable for inorganic phosphate determination.

CHAPTER IV

SUMMARY AND CONCLUSIONS

A. ANALYTICAL STUDIES

1. Calcium Determination

The studies in this thesis show that the ideal reagent for calcium determination in CSF was the modified CPC technique with 5M urea because the molar absorptivity (2.018×10^6 mol cm^{-2}) was higher than that of the unmodified CPC technique (1.5117×10^6 mol cm^{-2}). Chauhan and Sarkar (52) reported a value of 2.10×10^4 mol cm^{-2} with an ammonia/ammonium chloride buffer. Under semi-aqueous conditions, with DMSO in the system (53) together with the addition of urea (63), sensitivity was enhanced as indicated by a higher molar absorptivity.

CPC gave the highest molar absorptivity (2.018×10^6 mol cm^{-2}) compared to Calmagite (3.5766×10^5 mol cm^{-2}) or MTB (8.2528×10^5 mol cm^{-2}) studied under the same conditions. The precision (measured as the C.V.) for CPC was 1.40% as compared to that of Calmagite (4.98%) or MTB (8.60%). Thus, higher precision was obtained for CPC. A C.V. of less than 5% meant good precision and generally regarded as acceptable for clinical tests. CPC proved

to be highly selective and sensitive for calcium, and magnesium interference was eliminated with 8-hydroxyquinoline in the colour reagent (104). Potassium cyanide was added to DEA buffer to complex trace metals.

Calmagite and MTB both showed considerable interference from magnesium and their sensitivites (molar absorptivity) were not very high compared to CPC. However, as the molar absorptivity of MTB was higher than that of Calmagite, MTB would be more sensitive for calcium determination than Calmagite. Some magnesium interference was observed for both these reagents.

With the incorporation of DMSO in the system (53) there was an increase in sensitivity for CPC, as compared to previous results (52). The average recovery in serum was reported as 98.75% (52) while the average recovery in the present study was 99.5%. The addition of urea to the reagent increased the molar absorptivity (63) as confirmed in the present investigation. Due to the enhancement of its sensitivity, as little as 10 µL of sample could be used for each determination. The reagents are relatively stable and can be kept at room temperature for several weeks if the colour reagent and buffer are not mixed together. Cresolphthalein complexone would, therefore, be recommended for calcium determination in hospitals and clinical laboratories using spectrophotometric techniques.

2. Magnesium Determination

In the case of magnesium, Magon proved to be far more superior to Calmagite or MTB, not only due to its higher molar absorptivity (1.7652×10^6 mol cm^{-2}) but with regards to its selectivity, precision and stability. Calcium interference was eliminated with EGTA (90). The method gave results that correlated well with atomic absorption (90) and proved to be reliable. Other advantages to this method were the efficiency and simplicity of the tests with a rapid reaction time. The colour generated was stable for 24 hours. As the reagent was very sensitive for magnesium, a sample size of 10 µL would be sufficient for an accurate analysis. Mann and Yoe (83) reported an average recovery of 95.2% for magnesium while the present study gave an average of 99% for magnesium.

Next to Magon, Calmagite was more sensitive than MTB for magnesium determination as its molar absorptivity was 1.0059×10^6 mol cm^{-2} compared to 5.4302×10^5 mol cm^{-2} for MTB. Though high absorbances were obtained for magnesium (70,73), the reagent blank was too intensely coloured. Abernethy and Fowler (75) used a 55% Calmagite reagent while the present study employed a 40% Calmagite reagent (76). The reported C.V. for Calmagite with magnesium in day-to-day analyses on serum samples varied from 3-5% (73) while the average C.V. in the present study

was 3.84%. Calmagite was not particularly selective for magnesium as its molar absorptivity (1.0059×10^6 mol cm^{-2}) was almost comparable to 3.5766×10^5 mol cm^{-2} for its calcium complex.

MTB, on the other hand, was not sensitive for magnesium as its molar absorptivity (5.4302×10^5 mol cm^{-2}) was lower compared to Magon's (1.7650×10^6 mol cm^{-2}). The reagent was not very stable at room temperature and deteriorated after one or two weeks. The optimum conditions occurred only over a small pH range of 10.0 to 10.8 (78). The absorbances were very low when compared to the other two reagents. The degree of precision was considerably lower than for the others as illustrated by a C.V. of 8.63% compared to 3.84% for MTB and 1.15% for Magon. The use of ammonia buffer was a problem due to its odour which was obnoxious. All these disadvantages proved that MTB would not be as good as Magon for magnesium.

3. Phosphate Determination

For the determination of inorganic phosphate the phosphomolybdate technique was chosen because of its sensitivity and precision (100,102). The reported C.V. for day-to-day analyses on serum varied from 1.5-2.4% (100) while the C.V. for CSF was 1.96%. The recovery obtained by other researchers (100) varied from 95.4-97.9% for serum while the present study gave results that varied

from 100-107% for CSF. The reagents were fairly stable at room temperature even after a month. Though the method involved several additional steps, high accuracy and precision could be obtained as there were hardly any interferences from both proteins or other ions present.

B. CLINICAL STUDIES

1. Calcium Determination

CPC was found to be the best reagent for calcium determination in serum (53,104) as well as for CSF. The test was rapid and required no centrifugation or deproteinization of samples. Among the three reagents studied, CPC was the most sensitive (highest molar absorptivity), precise (C.V. 1.40%) and accurate (99.5% recovery) reagent for calcium. The method was highly selective for calcium with no interference from magnesium or other analytes and thus should be recommended for use in CSF.

2. Magnesium Determination

The best reagent for magnesium determination in CSF was Magon due to its high molar absorptivity. It proved to be superior to the others when studied under similar conditions. High accuracy (99% recovery) and precision (C.V. 1.15%) was obtained with CSF as compared to Calmagite or MTB. The method was direct and simple. Magon should thus be used for the determination of magnesium in CSF.

3. Phosphate Determination

The method involving the phosphomolybdate reaction was very specific and sensitive for phosphate determination (100,102). Results of the day-to-day analyses. (C.V. 1.96%) and recovery studies (103%) for CSF agreed well with those for serum (100). Pretreatment of samples was not required and colour formation took only a few minutes. The wavelength of maximum absorbance (700 nm) was ideal for phosphate as no other interfering ions absorb around that region (102). In view of these facts, the method should be recommended for inorganic phosphate determination in CSF.

Moreover, the analyses of all three analytes required only minute volumes of CSF (up to a maximum of 170 µL) which would be ideal even for pediatric samples. The tests were direct, rapid, sensitive, economical and practical in the clinical setting.

No centrifuging or complicated techniques were required as proteins or lipaemia were not major problems. These tests could easily be adapted for automation and they would be ideal for small clinical laboratories or hospitals with no atomic absorption spectrophotometers. These spectrophotometric methods should, therefore, be recommended for the analyses of calcium, magnesium and phosphate ions in CSF.

In conclusion, from all the reagents studied, CPC and Magon proved to be ideal for the determination of calcium and magnesium, respectively, while the phosphomolybdate method was useful in the determination of inorganic phosphate in cerebrospinal fluid.

APPENDIX

STATISTICS

C.V. Coefficient of variation is the standard deviation expressed as a percentage of the mean (105,106). It expresses relative precision over a wide range of values.

S.D. Standard deviation is the square root of the variance (107). It is inversely proportional to precision, thus, when S.D. increases, precision decreases.

V Variance is the sum of the squares of the deviations from the average divided by the degrees of freedom.

S.E.M. Standard error of mean normalizes the standard deviation.

FORMULAS

$$C.V. = 100\ S.D./\bar{x}$$

$$S.D. = \sqrt{\Sigma (x-\bar{x})^2/(n-1)}$$

$$S.D. = \sqrt{v}$$

$$V = \Sigma (x-\bar{x})^2/(n-1)$$

$$S.E.M. = S_{\bar{x}} = S/\sqrt{n}$$

$$r = \frac{N\Sigma XY - \Sigma X \Sigma Y}{\sqrt{[N\Sigma X^2 - (\Sigma X)^2][N\Sigma Y^2 - (\Sigma Y)^2]}}$$

$$y = bx + a$$

$$b = \frac{\Sigma (x-\bar{x})(y-\bar{y})}{\Sigma (x-\bar{x})^2} = \frac{N\Sigma XY - \Sigma X \Sigma Y}{N\Sigma X^2 - (\Sigma X)^2}$$

$$a = \bar{y} - b\bar{x} = \frac{\Sigma Y}{N} - b\frac{\Sigma X}{N}$$

$\bar{x}$ = mean

n-1 = degrees of freedom

x = individual value

n = runs

S = S.D.

r = correlation coefficient

v = variance

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